ROOTING MICROBES IN PLANT ADAPTATION:
THE EVOLUTION OF LOCALLY ADAPTIVE PLANT-MICROBE INTERACTIONS

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Ecology, Evolution, and Conservation Biology
in the Graduate College of the
University of Illinois Urbana-Champaign, 2023

Urbana, Illinois

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ABSTRACT

While an organism's phenotype can partially be attributed to heritable genetic variation, recent work has highlighted that phenotype is also partially influenced by an organism's interactions with its associated microbial symbionts. Indeed, all eukaryotic organisms are seemingly colonized by diverse communities of microorganisms that can frequently alter their host's phenotype through their various activities. However, while a number of microbial taxa that are important drivers of host phenotype have been identified, these interactions are often cryptic, with spatially and temporally variable effects on their hosts. This dissertation attempts to disentangle these interactions by examining drivers of both the host's and their microbial partners' evolution, specifically focusing on how their interactions may evolve to generate locally adaptive host phenotypes, using the interactions between plants and their root-associated microbes as a model symbiosis. I investigated multiple agents that may select on either the plant host or microbial partners, including the abiotic environment, as well as the reciprocal selection from each partner on the other. Overall, I found that these plant-microbe interactions could quickly evolve to provide locally adaptive benefits to the plants, though the drivers of this local adaptation varied. In one study I found plants were locally adapted to their historic environment only when provided with microbial partners, suggesting that these microbes were necessary for the evolution of the locally adaptive plant traits, with plants potentially evolving to adaptively interact with these microbes in their home environment. Additionally, across two experiments I found that plant-associated microbes had rapidly evolved to benefit plants in their current environment. In one of these, I found that plants were a strong selective agent on their microbes, driving the evolution of microbes that provided a locally adaptive benefit. In the other, I again

found these microbes evolved to provide a locally adaptive benefit to the plant, however, the plant was unnecessary in driving the evolution of these locally adaptive microbes. These results suggested that while microbes can explicitly evolve traits to benefit their hosts, these beneficial traits can sometimes simply be a byproduct of these microbes' own adaptation to their environment. Overall, this dissertation illustrates that host-microbe interactions are dynamic, with the potential to rapidly evolve in response to novel environments.

ACKNOWLEDGMENTS

I've only been able to complete this dissertation through the support and aid of dozens of mentors, colleagues, and friends. On the top of that list is my adviser, Dr. Anthony Yannarell, for guiding me through this entire process. You've been an incredibly patient adviser as I've bumbled my way through this process, giving me so much freedom to follow my research interests while also reining in some of my worse impulses. You've shaped the way I think about science and statistics. I also need to specifically thank my committee member, Dr. Katy Heath, for providing enormous amounts of intellectual and emotional support. You've supported me while I cried in your office and you're a role model for the type of scientist that I want to be; after this whole defense thing, can we be best friends? My other committee members, Dr. Angela Kent and Dr. James O'Dwyer, have challenged and guided me along the way. Angela…you, Sierra and I have probably spent too much time gossiping in lab, but I love it. I also would like to thank Dr. Jen Lau, for feedback on numerous projects. Your science inspires me, and I hope we can continue to collaborate. The GEMS Institute at University of Illinois has provided financial support for much of my work while also creating a stellar community to work on and think about symbioses. There are also many current and former members of the Kent, Yannarell, and Heath labs who have provided technical and intellectual support, inspiring discussion about science, as well as feedback on presentations, ideas, and writing: Alonso Favela, Joe Edwards, Gabe Price, Sierra Raglin, Isaac Klimasmith, Sandy Simon, Kristen Ellis, Ilana Zeitzer, Noah Brown, Sierra Bedwell, Izzy Lakis, David Vereau, Ivan Sosa, Meghan Blaszynski, Rebecca Batsone, Abdel Halloway, and Mario Céron Romero.

I can’t name everyone in this short space, but there have been countless friends who have supported me through this process. Like many graduate students, when I moved to Illinois I felt incredibly isolated, not knowing anyone within several hundred miles of the University. I’d never lived in a place with so little topography and too much corn and I immediately questioned all of my decisions. But over the course of these past few years, I’ve built an amazing community and friendships that will last a lifetime. Among these include my ride-or-die labmate, Sierra Raglin, who I’ve gossiped with my whole 5 years here and who also successfully defended her dissertation right before me. I’ve laughed and cried with you, and you may be my research soulmate. You’re an amazing, inspiring scientist and we’re going to collaborate forever. Additionally, to Alex Riley, Rob Walls, Madeline Herman, Natalie Broadhurst, Chris Curtis, and Sierra Bedwell, for being some of my best friends and killer belay partners. You got me touching grass, climbing real rocks, and helped take care of my greenhouse plant babies. Also, to Sierra Pérez, for being my rock this entire time. We hated each other when we first interviewed at Illinois, and now 5 years later, you bring me back to reality. I can’t imagine my life without you in it. I also need to thank several of my family members for their continual support. My brother, Nathan, with whom I’ve never been able to stop talking science, to the point that we probably annoy the rest of our family. You’ve helped me refine my ideas and collected samples for one of my chapters. To my parents, Rodger and LaRee, for listening to my manic science rants, while also instilling a love and respect for this planet. I don’t know if I would have become an ecologist if it wasn’t for the gardening with Mom in the summer and the many camping trips with Dad in the Cascades. And finally, to my partner, Jack, for enduring endless “science talk” and snarkiness and yet still emotionally anchoring me. You’ve helped me see the light in a dark place.

To my mother for encouraging me to find my own path

And to Jack, for exploring this beautiful world with me

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CHAPTER 1: ROOTED IN HISTORY? REVIEWING THE CONTRIBUTION OF PLANT-MICROBE COEVOLUTIONARY DYNAMICS TO LOCAL ADAPTATION

INTRODUCTION

While an organism's phenotype can partially be attributed to heritable genetic variation (Kaprio 2012, Lind et al. 2018, Young 2019), recent work has suggested that a portion of the unexplained variation in phenotype could be attributed to an organism's interactions with its associated microorganisms, also known as their microbiome (Sandoval-Motta et al. 2017, Simon et al. 2019). Indeed, all eukaryotic organisms are colonized by complex and diverse communities of microbes, including bacteria, fungi, archaea, and viruses, who alter their host's phenotype through their various activities and traits (Friesen et al. 2011, Chaston et al. 2014, Petipas et al. 2021). In an effort to uncover the microbial contribution to phenotype, there has been an abundance of research identifying the key microbial taxa that significantly contribute to their host's phenotype, as well as the drivers of microbiome composition (Wagner et al. 2014, Mancabelli et al. 2017, Brunel et al. 2020). While such research directions have certainly been fruitful, a perhaps understudied and important component in this host-microbial interaction is the selective pressures upon the traits that underly these interactions.

Indeed, given the short generation time for many microbial taxa, a host's associated microbes can rapidly evolve, potentially altering the outcome of the host-microbe interactions on ecologically relevant timescales (Garud et al. 2019, Li et al. 2021). Similarly, given the strength of selection these microbes can exert on their hosts, a host population's interactions with its microbiome are not static, with host populations evolving in response to microbially-driven selective pressure. For example, pathogens/parasites drive a coevolutionary arms race between

their hosts' immune systems and their own infection strategies (Koskella and Lively 2009, Han 2019, Minias and Vinkler 2022). Consequently, the coevolution of these host-microbe interactions, as well as the context dependency of these microbes (Hoeksema 2010, Nelson et al. 2018, Batstone et al. 2020b), may rapidly shift any given interaction along a continuum between parasitism and mutualism (Hirsch 2004, Karst et al. 2008, Drew et al. 2021).

While there have been a number of microbial taxa identified as important drivers of host phenotype (Zaura et al. 2009, Tsukuda et al. 2021), their evolution and context dependency can lead to cryptic interactions, with spatially and temporally variable effects of these taxa (Weeks et al. 2007, Li et al. 2021). As such, this short review will focus on the selective drivers of host-microbe interactions, specifically focusing on plant-microbial interactions as a model system. I first briefly review mechanisms through which plants and their associated microbes can impact their partner's fitness and phenotype. I will then discuss mechanisms that may drive the evolution of these interactions, identifying areas of future study, and linking specific areas to the focus of my dissertation work. Throughout this process I will consider the selective drivers of both the host plant as well as their associated microbes. Ultimately, unraveling the impact of microbes on host phenotype will help dissect the contributions of host genetics, environment, and microbial interactions, thereby advancing our understanding of host traits.

BACKGROUND TO PLANT-MICROBE INTERACTIONS

In both this review, as well as throughout this dissertation, I specifically focus on the plant microbiome. I argue here that plants and their microbiomes are a model system for studying the coevolutionary dynamics between hosts and their associated microbes. Firstly, due to their frequent small size and short lifespan, many plants are amenable to use in experimental settings,

allowing for large amounts of data to be rapidly generated. Experiments in this system can often be tuned to the specific questions at hand as the soil abiotic and microbial environments may be easily manipulated. Specific model plants, including *Zea*, *Arabidopsis*, *Medicago*, and *Lemna*, to name a few, have extensive genetic resources available, which can also facilitate the study of the genomics of these interactions (Strable and Scanlon 2009, Koornneef and Meinke 2010, Gasch et al. 2016, Krishnakumar 2019, Acosta et al. 2021). Additionally, due to the sessile nature of plants, there may be strong eco-evolutionary feedbacks between the plant hosts and their associated microbes (TerHorst and Zee 2016, Van Nuland et al. 2016); namely, populations of microbes in the soil that associate with and are selected upon by one generation of plants may be likely to associate and be selected upon by the next generation of plants. This may contrast with the interactions between various animals and their resident gut microbiota; as those host organisms are mobile, microbes are acquired and deposited across the host's landscape, potentially minimizing locally adaptive host-microbe interactions (though see Houwenhuyse *et al.*, 2021).

Plant-associated microbes can impact plant phenotypes through a variety of mechanisms (Friesen et al. 2011), and colonize multiple plant compartments in complex and functionally diverse microbial communities (Berg et al. 2014, Compant et al. 2019). The zone immediately surrounding the plant root, classified as the rhizosphere, can be colonized by thousands of active microbial taxa due to the release of photosynthetically-derived carbon in the root zone (Prashar et al. 2014, Brunel et al. 2020). Microbes also colonize the interior of the plant, classified as endophytic microbes, colonizing both intra- and intercellularly, in both above- and belowground tissues (Bacon and White 2016). Across their various compartments, these plant-associated microbes can act as tiny biochemists, synthesizing and transforming a variety of bioactive

compounds that interact and influence the plant. For example, all major classifications of plant hormones have been observed to be produced by plant-associated microbes, including auxins, cytokinins, gibberellins, abscisic acid, and ethylene (Spaepen 2015). Hormones produced by these microbes can interact with the plant, altering plant resource allocations, including changes to above- and belowground architecture (Dodd et al. 2010, Spaepen 2015). Similarly, these microbes can produce a diverse array of volatile organic compounds (VOCs), which can interact with the plant, altering plant gene expression, while also acting as chemical cues for other organisms (Bitas et al. 2013, Fincheira et al. 2021). For example, VOCs produced by *Fusarium* in the soil environment increased plant root length, while microbes colonizing floral nectaries can produce specific VOCs that attract pollinators (Schenkel et al. 2018, Vannette 2020, Cullen et al. 2021). Endophytic microbes can produce a variety of alkaloid compounds that alter a plant's internal biochemistry, with the potential to protect the plant from pathogens or herbivores, while also altering plant gene expression (Zhang et al. 2012, Daniel et al. 2017).

In addition to their role as tiny biochemists, these plant-associated microbes can frequently influence plant nutrient acquisition (Fierer 2017, Kumar et al. 2018, Pantigoso et al. 2022). As microbes are the primary drivers of biogeochemistry and nutrient transformations in the soil (including, nitrogen, phosphorus, iron, sulfur, etc.), these plant-associated microbes can either increase or decrease nutrient availability to the plant (Fierer 2017, Crowther et al. 2019, Sokol et al. 2022). Moreover, many plants have formed specialized symbiotic interactions with specific microbial taxa that aid in the acquisition of nutrients. For example, the majority of plant species can associate with mycorrhizal fungi (Brundrett 2009, Bueno et al. 2017). These fungi form dense hyphal networks throughout the soil, leveraging their large surface areas to effectively scavenge for soil phosphorus, which they exchange with the plant for

photosynthetically derived carbon (Bolan 1991, Genre et al. 2020). Leguminous plants form a similar symbiotic with rhizobia bacteria, which colonize plant root nodules. Therein, these bacteria fix atmospheric nitrogen into a biologically available form, which they exchange with the plant for plant carbon (Udvardi and Poole 2013, Poole et al. 2018). Perhaps receiving the most attention as a result of plant-based industries are the abundance of pathogenic/parasitic microbes that colonize plant, having frequently specialized to exploit the plant for its resources, causing plant disease and increasing plant mortality (Doehlemann et al. 2017, Lefeuvre et al. 2019).

While the above description of potential plant-microbe interactions is certainly not a comprehensive list of the various microbial activities that can influence plant phenotype, it illustrates the broad diversity of their interactions as well as their potential impacts on the plant. As these microbes alter plant fitness and phenotype, and these interactions can be context dependent, these microbial interactions inherently alter the plant's potential niche, either expanding or contracting the niche depending on the relative benefit or detriment to the plant in a given environment (Peay 2016, Lemoine et al. 2020). For example, these microbial interactions can increase or decrease plant resource acquisition or stress tolerance, thus potentially modulating access to new environments. A classic example of the microbial-influenced plant niche is the plant interaction with mycorrhizal fungal, which have long been implicated in the plant adaptation and transition to land (Pirozynski and Malloch 1975, Brundrett Mark C. 2002, Feijen et al. 2018). Paradoxically, beneficial interactions might also limit a plant's potential niche. For example, some plant hosts may be fully obligate on their microbial partner to provision specific resources. This dependency on the interaction may generate a potential "two-body problem", as the abiotic limitations of one partner will limit the other partner (Wernegreen

2012, Duffy and Johnson 2017). Given this diversity of potential interactions, understanding the evolution of these interactions will have clear impacts on plant phenotype, and facilitate understanding of plant adaptation and distribution across a variety of habitats.

MICROBIAL EVOLUTION

While this review takes a plant-centric view, focusing on the microbial impact on plant phenotype, the effect of the plant on microbial fitness and evolution is essential in evaluating these plant-microbe interactions. Plant-associated microbes have frequently been viewed as passively dependent on the plant and an accessory to its phenotype, ignoring the selective pressures driving microbial evolution (Klein et al. 2021). However, I emphasize that while plants and microbes may associate with one another, microbial evolution is frequently independent of the plant, driven to increase microbial fitness; any microbial impact on the plant is a product of microbial traits increasing microbial fitness. In this light, below I review some of the selective agents on these plant-associated microbes, as well as how these agents may interact to drive the evolution of plant-associated microbial traits.

These plant-associated microbes can face multiple agents of selection, ranging a variety of environments. For example, many plant-associated microbes can persist in a free-living state, independent of the plant, in the bulk soil environment. Many of the microbes in this environment are significant drivers of biogeochemistry and decomposition. While this environment is frequently resource-poor (Whalley et al. 2005), many of these same microbes can colonize the plant rhizosphere, a comparatively resource-rich environment, with a high degree of microbial activity, as a consequence of plant's depositing/exuding a significant portion of their resources into this zone (Berg and Smalla 2009, Zhalnina et al. 2018, Qu et al. 2020). Specifically, plant

roots exude a variety of forms of labile carbon, including polysaccharides, organic acids, amino acids, etc. (Hennion et al. 2019), which act as critical resources for many of the microbes in this zone. Moreover, while the rhizosphere is rich in resources, the plant's deposition of these resources can physically and chemically alter this environment, acting as a strong selective agent on these plant-associated microbes. For example, rhizodeposition can alter the pH and redox potential of this zone (Nye 1981, Brunel et al. 2020, Munoz-Ucros et al. 2021), while also excreting a variety of compounds, including flavanols, phenols, etc. (Glass 1976, Weston and Mathesius 2013) that can inhibit the growth of specific microbial taxa (Berg and Smalla 2009, Baetz and Martinoia 2014, Zhalnina et al. 2018). Plant compounds like flavones stimulate microbial biofilm formation, altering the polysaccharide matrices within which microbe-microbe interactions occur (Wang et al. 2022). The high microbial activity in this zone can also deplete oxygen in this environment, potentially selecting for microbes with anaerobic capacity (Qu et al. 2020, Pantigoso et al. 2022). The microbial activity in this zone is a strong driver of the microbial impact on the plant (Qu et al. 2020, Pantigoso et al. 2022). A subset of the microbes that colonize the rhizosphere zone can go on to endophytically colonize the interior of the plant roots (Berg et al. 2014, Compant et al. 2019). Endophytic colonization first requires that these endophytic microbes must bypass the root epidermis, circumvent or tolerate the plant immune and chemical defense, while also obtaining nutrition from the plant tissue (Herre et al. 2007, Arnold et al. 2009).

Microbes must be able to pass through these various and differing selective filters to colonize these various compartments on the plant. As the microbial traits that allow them to persist in these environments may interact with and influence plant phenotype, these selective plant filters may drive plant phenotype through selection on their microbiome. As a significant

number of the microbes that influence plants are immediately surrounding the plant in the rhizosphere or endophytically colonize the plant, I will initially focus on these microbe's selective agents.

As the microbial impacts on the host can range on a continuum from beneficial to negative, what selective pressures will drive the evolution of rhizosphere or endophytic microbial traits that are beneficial to the plant? While many of the microbial traits previously described can certainly benefit the plant hosts, from hormone production, to resource acquisition, to defense against pathogens, these traits can often be costly. Indeed, theory predicts that microbial traits beneficial to the plant will be selected for when these same traits simultaneously increase microbial fitness (O'Brien et al. 2021). Namely, these beneficial microbes may arise when there is an alignment between plant and microbial fitness, where increases in one partner's fitness increase the fitness of the other partner.

There may be various plant-based mechanisms that align plant and microbial fitness. For example, through shared transmission, also known as vertical transmission, microbial symbionts are transferred directly from the parent to the offspring, aligning partners' fitness as the reproduction on the host guarantees the reproduction of the symbiont. Consequently, symbiont traits that benefit the host fitness will also benefit symbiont fitness and may therefore be selected for. Vertically transmitted plant symbionts can be observed in the interactions between the aquatic plants in the *Azolla* genus and their symbiont, the cyanobacteria *Anabaena.* The leaf tissues of *Azolla* are intercellularly colonized by *Anabaena*, that, like rhizobia, fix and provide atmosphere nitrogen for their host plant. These symbionts are transmitted from one plant generation to the next as *Azolla* plants can reproduce through vegetative fragmentation, allowing the cyanobacteria to move directly to the next generation, while also colonizing the reproductive

sporocarps when the plant reproduces sexually (Lechno-Yossef and Nierzwicki-Bauer 2002). It is unclear how common vertical transmission is among plant-associated microbes, but it is one driver that could align microbial fitness to plant fitness and strengthen their coevolutionary response (Sachs et al. 2004, Leigh 2010).

Vertical transmission contrasts with horizontal transmission, likely the primary mechanism of microbial colonization, wherein microbes are acquired at the start of each plant generation. The alignment of microbial and plant fitness is not inherent when horizontally transmitted, as when vertically transmitted, though there are potential mechanisms that align fitness. For example, the resources exuded into the rhizosphere by the plant can benefit these microbes in those zones, opening avenues for potential alignments in partner fitness; indeed, as microbes benefit a plant, increases in overall plant health could lead to increases in plant-provisioned resources to microbes, increasing their fitness. Examples of this fitness alignment are especially well studied in the legume-rhizobia symbiosis, as well as the plant-mycorrhizal symbiosis. In these systems, significant coevolution between these partners has developed various plant-based mechanisms through which the plant can ‘identify’ the quality of the rhizobia and mycorrhizal partners, preferentially associating and allocating resources to high-quality partners (Kiers et al. 2011, Westhoek et al. 2017, Oono et al. 2020). This system of plant rewards ensures that microbial traits beneficial to the plant are also beneficial and rewarding to the microbe, ensuring alignment in plant and microbe fitness. Without these mechanisms, it may be unclear whether these beneficial microbial traits would be selected for. Indeed, both the exchange of nitrogen and phosphorus to their plant hosts involve a series of energetically expensive processes, and the primary benefit arising from rewards received from the plant. Without a plant preferential allocation mechanism, microbial partners that do not benefit the

plant should be selected by outcompeting beneficial microbial partners, effectively ‘cheating’ the interaction (Jones et al. 2015).

Outside of these specialized and well-studied interactions symbioses, there may still be mechanisms for aligning plant and microbial fitness. Preferential allocations of root resources to specific root zones through adaptive foraging could be the basis for fitness alignment. Plants may direct root resources and growth towards microbes that provide resources, protection from pathogens, and other benefits, increasing both plant and microbial fitness. Alignment may also be maintained through forms of pseudo-vertical transmission. For example, endophytic microbes can frequently colonize seeds produced by a plant before their dispersal (Gundel et al. 2012, Cope-Selby et al. 2017), partially aligning plant and microbial fitness. To understand the evolution of microbial host-associated traits, we need to expand beyond model symbioses, determining the selective drivers on their host-associated traits.

Even in the absence of plant mechanisms to explicitly align plant and microbial fitness, as described above, microbes that benefit the plant may still persist. There are a variety of microbial traits that increase microbial fitness but incidentally benefit the plant as a byproduct of their activity. For example, under droughted conditions there may be selection for microbes that produce biofilms, as this trait can prevent microbial desiccation (Lennon et al. 2012). While this is a microbial trait that is selected for the explicit purpose of increasing microbial fitness in drought, it may incidentally facilitate plant drought tolerance as the biofilms can coat plant roots, also preventing their desiccation and osmotic pressure (Naseem et al. 2018). Similarly, highly competitive microbial communities may select for microbes that can produce antibiotics or antifungals, to use in a form of chemical warfare against competing microbes (Williams and Vickers 1986, Wiener 1996). However, the production of these compounds can simultaneously

be beneficial to the plant by defending the plant against pathogens (Bednarek and Osbourn 2009). These byproduct mutualisms may lead to an alignment between the microbial and host fitness; however, this alignment is an incidental byproduct of the microbial trait and has not been selected for as a product of the plant-microbe interaction. These byproduct mutualisms may be the target of future coevolutionary interactions with the plant, transitioning to explicit mutualisms similar to rhizobia and mycorrhizal symbiosis (Harcombe et al. 2018). For example, the rhizobia mutualism has been speculated to have evolved from free-living N fixers in the soil. As plants receive strong benefits from the N byproduct of these microbe's activities, plants may have evolved mechanisms to explicitly direct root carbon resources towards effective N-fixers (Frederickson 2013). Investigating the origins of many plant-associated microbial traits and the selective drivers of these microbial byproduct traits, independent of plant selection, may further connect microbial evolution with plant phenotype. To this end, in chapters three and four of this dissertation, I investigated the role of byproduct mutualisms in mediating adaptive plant responses.

While many of the microbial taxa that impact plant phenotype colonize the rhizosphere or endosphere, some microbes that do not colonize the plant can still influence plant phenotype. For example, in the bulk soil environment, outside of zones colonized by plant roots, there are an array of free-living microbes in the soil involved in a variety of biochemical and nutrient cycling processes (Fierer 2017). The activities of these microbes, even for those not associated with a plant, can alter the physical soil environment within which the plant lives, having indirect impacts on the plant through changes in soil nutrition, carbon availability, pH, redox, etc. While these microbes' traits can potentially influence plant phenotype, their evolution may be independent of interactions with the plants. These microbes can again be seen similarly as a

byproduct symbiosis and may not necessarily be restricted to single plant hosts. Understanding the selective drivers of the microbe's responsible for these functions across a variety of environments may aid in connecting these microbe's evolution to plant phenotype.

PLANT EVOLUTION

While above I explored the evolution of plant-associated microbes, here I additionally review how these microbes can act as agents of selection on plant evolution. Indeed, there is mounting evidence that microbes can act as selective agents on their host plants, driving the evolution of hosts locally adapted to the locally available microbial partners (Grillo et al. 2016, Amandine et al. 2022). Coevolution between these partners may have a variety of impacts on the outcome of plant-microbe interactions and how these interactions influence plant phenotype.

For example, given the potential for beneficial interactions on plant fitness, there may be selective pressure for plants to preferentially interact with specifically beneficial microbial taxa. Indeed, as reviewed earlier, in a number of specialized plant-microbe symbioses, including legumes and other mycorrhizal plants, plants use an array of chemical signals to recruit their respective symbionts (García-Garrido et al. 2009, Streng et al. 2011, Garcia et al. 2015, Ghantasala and Roy Choudhury 2022). These signals ensure the plant is colonized by the 'right' taxa of rhizobia and fungi. These signals have likely been selected through the millions of years of coevolution between these partners. Similarly, some hosts may ensure continued access to their specific microbial partners by evolving specialized compartments for interacting with their partners. For example, *Anabaena* symbionts in *Azolla* are housed in a specialized extracellular compartment on the leaf, used solely for their colonization, wherein they are enclosed with a mucilaginous material (Lechno-Yossef and Nierzwicki-Bauer 2002). Similar interactions can be

found in many insect taxa, which have evolved specialized bacteriocyte cells that house nutritional bacteria symbionts (Skidmore and Hansen 2017).

Outside of these specialized and well-studied symbioses there may have also been selection for plant traits to preferentially interact with beneficial microbes. For example, as reviewed earlier, the plant environment is highly selective for microbial symbionts. Through their exudation patterns and activities, plant roots alter the physical, nutritional, chemical, and redox environments immediately surrounding their roots while the interior plant environment includes a variety of chemical and immune signals. The plant traits governing these environments act as filters on the microbes interacting with the plant, potentially allowing for only specific microbial taxa to colonize. Given the significant impact these microbes may have on plant fitness and phenotype, there may be selection for plant traits that engineer root ecosystems hospitable to specific beneficial microbial taxa while excluding those that do not benefit the plant (Ricks and Koide 2019). Indeed, in chapter two of this dissertation, I investigated if a plant's interactions with its broad root microbial community can provide a locally adaptive response, which may indicate at selection for plant traits guiding the microbial interactions. Similarly, recent work has suggested that there may have been selection for plants inhibiting microbial nitrification (Favela et al. 2021). Nitrification, or ammonia oxidation, is the microbial transformation of ammonia, a readily available and largely immobile form of nitrogen, to nitrate, a soluble form of nitrogen that can be easily leached from the soil system (Stein et al. 2016). Nitrification is driven by a phylogenetically distinct group of nitrifier bacteria (Srivastava et al. 2016). Under low nitrogen conditions, plants largely benefit from low activities of nitrifiers as their activity can lead to the loss of nitrogen from the system, while under high nitrogen conditions, their activity may have little impact on the plant as the plant is likely not nitrogen

limited. Consequently, the nitrogen environment may drive the evolution of plant traits associated with these nitrifying bacteria. Indeed, Favela and colleagues (2021) recently showed that modern varieties of maize, which have largely been bred under high nitrogen conditions of the green revolution, have a significantly higher nitrification activity, compared to more historical varieties bred in lower nitrogen conditions that suppress nitrifier activity. This work, as well as others, presents exciting new avenues for the investigation of the evolution of various plant traits that may indirectly influence plant phenotype through their interactions with plant-associated microbes.

Microbial interactions with a benefit to the plant may also drive the loss of function in their plant hosts. These beneficial interactions often replace plant functions, including nutrient acquisition or stress tolerance. Given consistent selection from these microbial interactions, maintaining plant traits that these microbial interactions have replaced may be costly, potentially driving the loss of these plant traits. For example, leguminous plants rely on rhizobia for nitrogen acquisition and have almost entirely lost their capacity to scavenge nitrogen from the soil without their rhizobia partners. Consequently, some plant traits may not solely be a result of the plant's own heritable genetic variation, but are reliant on the complex interaction with their locally available microbial symbionts.

I note that not all the plant traits that impact their associated microbes may necessarily have evolved for the express purpose of impacting the plant's microbes. Many plant traits that influence the plant-microbe interactions can be pleiotropic and consequently have multiple selective agents. For example, jasmonic acid (JA) is a plant hormone that is involved in the plant's response to stress, while also influencing microbial recruitment in the plant (Kniskern et al. 2007, Carvalhais et al. 2017). Consequently, abiotic stress may select for specific JA

biosynthesis and regulation genes, which could have cascading effects on microbial interactions and thus how these microbes impact plant phenotype. When evaluating plant traits associated with microbes, not all may necessarily be adaptive with respect to the microbial interaction.

CONCLUSION

While there has been a significant body of work highlighting the importance of host-associated symbionts in determining host fitness, the drivers of these symbiotic microbes' evolution remain poorly understood. Herein I have briefly reviewed plant-microbe interactions, including the various mechanisms through which they can influence the plant, as well the selective drivers of these interactions' evolution. Integrating the molecular coevolution of both the host and their associated microbial symbionts will provide insight into the evolutionary drivers behind the microbial contribution to host fitness while future work should expand to numerous new plant and microbial systems beyond the model systems to fully capture the evolutionary ecology between plant and microbial partners. To this end, in this dissertation I worked towards linking the selective pressures on a plant's microbiome with plant phenotype.

In my second chapter, I first investigated the microbe's selective role on plant evolution. To this end, I collected genotypes of *Bromus tectorum*, an annual grass, from a variety of salinity habitats. I then examined whether these plants' interactions with these microbes facilitated an adaptive response to their home salinity environment, finding that patterns of local salinity adaptation only emerged when these plants were provided with microbes with which to interact. These results may indicate the plant's associated microbes have been significant agents of selection in these plants' evolution.

In my other two data chapters, chapters three and four, I instead examined how microbes may evolve new traits that impact plant phenotype. I specifically leveraged the rapid generation times of microbes to use an experimental evolution approach, investigating various selective factors on these microbes' evolution. I broadly investigated the role of the plant in selecting for microbes with an adaptive benefit to the plant. To this end, in both chapters I evolved microbes under variable moisture environments (wet or dry), and either with or without a plant, and then inoculated these plants back onto plants under these conditions. This approach allowed me to determine if adaptive microbes arose as a product of plant selection or whether these adaptive benefits were a byproduct of microbes adapting to a given environment. Overall, I found mixed results. In my fourth chapter, I specifically evolved rhizobia symbionts and found that beneficial traits only evolved when interacting with a plant, suggesting that plants were significant selective agents in driving the evolution of the beneficial microbial traits. However, in my third chapter, I evolved a whole soil microbial community and found that adaptive microbial communities arose even without a plant present, suggesting these adaptive traits were a byproduct of these microbes' own evolution. These results highlight that these beneficial microbial traits can arise across a variety of mechanisms.

Overall, in this dissertation I show that plant-microbe interactions, including both the plant and microbe, may rapidly evolve in response to various abiotic factors. These abiotic environments frequently selected for microbial interactions that provided a locally adaptive benefit to the host, though the drivers of this adaptation varied.

CHAPTER 2: PATTERNS OF PLANT SALINITY ADAPTATION DEPEND ON INTERACTIONS WITH SOIL MICROBES[1]

ABSTRACT

As plant-microbe interactions are both ubiquitous and critical in shaping plant fitness, patterns of plant adaptation to their local environment may be influenced by these interactions. Identifying the contribution of soil microbes to plant adaptation may provide insight into the evolution of plant traits and their microbial symbioses. To this end, I assessed the contribution of soil microbes to plant salinity adaptation by growing ten populations of *Bromus tectorum*, collected from habitats differing in their salinity, in the greenhouse under either high salinity or nonsaline conditions, and with or without soil microbial partners. Across two live soil inoculum treatments, I found evidence for adaptation of these populations to their home salinity environment. However, when grown in sterile soils, plants were slightly maladapted to their home salinity environment. As plants were on average more fit in sterile soils, pathogenic microbes may have been significant drivers of plant fitness herein. Consequently, I hypothesized that the plant fitness advantage in their home salinity may have been due to increased plant resistance to pathogenic attack in those salinity environments. Our results highlight that plant-microbe interactions may partially mediate patterns of plant adaptation as well as be important selective agents in plant evolution.

[1]This work has been accepted to The American Naturalist, and is currently pending print: Ricks KD, Ricks NJ, & Yannarell AC. Patterns of plant salinity adaptation depend on interactions with soil microbes. The American Naturalist (2023). https://doi.org/10.1086/725393

INTRODUCTION

As plants can be widely distributed in space, encountering a variety of environments, it is often expected that different populations may be adapted to their local environments, with plant genotypes having higher relative fitness in their home environments than plant genotypes from foreign environments (for example, Figure 2.1A; Blanquart *et al.*, 2013). While such adaptation to the plant's local environment is relatively common (Hereford 2009), the traits underlying driving this adaptation may be unclear. For example, given there can often be stark contrasts in the abiotic environments between populations, it may be assumed that these patterns of local adaptation are the result of the abiotic environment selecting on genetically-based plant phenotypes that are adaptive to that abiotic environment. However, plants are colonized by large communities of microorganisms, and these microbes -- through their various activities -- can influence plant fitness and phenotype (Friesen et al. 2011, Fitzpatrick et al. 2019, Trivedi et al. 2020). In addition, plants can provide resources and act as habitats to their associated microbes (Broeckling et al. 2008, Berg and Smalla 2009), leading to potential feedbacks between microbial and plant fitness. Consequently, these interactions may facilitate both plant and microbial partners to act as significant selective agents upon the other partner. Therefore, a plant's fitness and traits in a given environment are not necessarily a product of its own evolution alone (Partida-Martínez and Heil 2011) but rather reflect a series of complex ecological and evolutionary interactions between a plant's genome, abiotic environment, and associated microbes. Patterns of plant local adaptation could therefore be influenced and/or driven by these plant-microbe interactions.

Understanding the microbial contribution to these patterns of plant local adaptation will facilitate a better understanding of the evolution and impact of these microbial symbioses on

their host's ecology and evolution. While host-microbe interactions are ubiquitous, with all eukaryotic organisms apparently being colonized by complex communities of microbes (Gordon et al. 2013, Simon et al. 2019), the evolutionary context behind many of the interactions is unknown, whether these interactions are adaptive, or how these interactions may shape the trajectory of each partner's evolution. By partitioning the patterns of plant adaptation to adaptation to the abiotic and biotic components, we may be able to bridge this gap and consequently provide insight into whose traits (the plant's, the microbes', or a combination of both) confer relative fitness benefits to plants between different environments, and thus the evolution of and selective pressures on these plant-microbe symbioses.

Plant-microbe interactions could be important in contributing to these patterns of plant local adaptation through a number of ecological- and evolutionary-based mechanisms. For instance, patterns of plant local adaptation could be driven by plants adapting to their abiotic environment through selection on their microbial interactions. Plant-microbe interactions can have a heritable component, determining the relative abundance of specific microbial taxa, as well as those taxa's impact on plant health (Aira et al. 2010, Gehring et al. 2017, Walters et al. 2018, Bergelson et al. 2019). Consequently, there may be selection for specific plant-microbe interactions dependent on the abiotic environment. For example, the mycorrhizal colonization of root systems is a heritable trait (Wang et al. 2010, Patterson et al. 2019, Anthony et al. 2020), and increased mycorrhizal colonization can increase plant drought tolerance (Al-Karaki et al. 2004, Gehring et al. 2017, Liu et al. 2018). Therefore, droughted environments may select for plants with increased association with mycorrhizal fungi, which enhances plant drought tolerance and thus indirectly produces a plant phenotype that indirectly confers a drought advantage even though mycorrhization is the direct phenotype under selection. While this is another form of

selection on the plant to the abiotic environment, here the plant depends critically on the presence of microbes to manifest the adapted trait to that abiotic environment. Patterns of plant local adaptation could also be driven by adaptation to their local microbial partners, independent of adaptation to the abiotic environment. Coevolution between plants and their microbial partners could result in plants with a "home field advantage" when interacting with mutualistic microbes that shared their historical habitat. Indeed, some prior work has suggested that a plant's fitness can be maximized when matching plants and microbes from the same habitat, which may be the result of either the microbes or the plant adapting to their respective partner (Johnson et al. 2010; Batstone et al. 2018; but see also Reinhart et al. 2003; van der Putten 2010). Thus, when a plant is grown in its home environment, it is more likely to interact with its historic microbial partners with whom local plant genotypes have recently coevolved, potentially providing a fitness benefit to the plant. These fitness benefits to the plant would be the result of plants adapting to the microbial populations from their local habitat, but in the reciprocal transplant experiments often used in examining local adaptation, this could easily be misattributed to adaptation solely of the plant to the abiotic environment.

Indeed, reciprocal transplant experiments are commonly used to study location adaptation to the abiotic environment, but their use can limit our ability to study the role of microbes in influencing these patterns of plant adaptations. A plant's local soil microbes will generally not accompany it into the transplanted environment, and this can place these plants in an unrealistic or sub-optimal microbial context. Moreover, these experiments rarely include a sterile control, where plants do not interact with any microbes, making it difficult to separate the abiotic and biotic components of plant adaptation. While these experiments inherently capture much of the abiotic variation that is important for plant adaptation, it is harder to manipulate the biotic

microbial environment, and this is especially true in common garden field trials (though see Petipas *et. al* 2020). To this end, to understand the evolution of these plant-microbe interactions, I examined their contributions to plant adaptation using a greenhouse experiment where the microbial inoculum could be controlled. Specifically, I collected seeds from populations of the grass *Bromus tectorum* from both high and low salinity habitats and generated microbial soil inocula from those same habitats. I evaluated the adaptation of these plant populations to their home salinity environments by growing plants in the greenhouse under high salinity and nonsaline conditions, both with microbes originating from saline and nonsaline environments and in sterilized soils.

Our aim in this contribution was to isolate the interactive biotic and abiotic components of plant adaptation to their home salinity environments. I compared plant salinity adaptation between sterile and live soil conditions, where sterile conditions represent a test of solely the abiotic adaptation, while live conditions represent a test of both abiotic and biotic adaptation (Figure 2.1B, 2.1C). As such, in this approach, significant impacts of the interactions between the salinity and microbe treatments on plant fitness across populations would indicate at the importance of plant-microbe interactions in influencing plant adaptation through a variety of mechanisms. Moreover, by comparing plant adaptation across several live microbial inocula, with inocula that originated from either saline or nonsaline environments, we may evaluate the role of specific microbes in influencing plant salinity adaptation; specifically, the importance to plant adaptation of matching microbes from a given abiotic environment with that abiotic environment as well as plants from that same abiotic environment (e.g., saline microbes with saline plants in a saline environment).

MATERIALS AND METHODS

Plant material collection

Bromus tectorum L. was introduced to North America in the late 1800s, where it subsequently invaded large portions of the American Southwest (Mack 1981, Knapp 1996). It has rapidly adapted to a variety of habitats, including highly saline habitats such as playas and salt deserts (Leger et al. 2009, Scott et al. 2010, Merrill et al. 2012). Moreover, *Bromus tectorum* is an annual, cleistogamous, plant with minimal outcrossing (Novak and Mack 1993), model traits for greenhouse work. Populations of *Bromus tectorum* from various salinity environments, therefore, presented an ideal natural experiment for examining the contribution of plant-microbe interactions to plant salinity adaptation.

In June 2019, I collected seeds from 10 populations of *Bromus tectorum* from habitats that varied in their salinity, spread throughout northern Utah (Table A.1, Figure A.1). Of these, 5 populations were collected from habitats classified as saline, indicated by high electrical conductivity soils, and 5 populations were collected from habitats classified as nonsaline, indicated by low electrical conductivity soils measured at the time of seed collection (Figure A.2). While there may be other environmental differences between these populations that contribute to plant adaptation to their local environment, the soil conductivity observed in the saline habitats is toxic to many plants and therefore likely a strong selective agent on the plants. Therefore, I chose to focus on plant adaptation to their home salinity environment as the focal treatment for our experiment.

Two saline populations were collected on a large playa surrounding the Great Salt Lake. The seeds from 2 additional saline populations were collected from a playa on the south side of Utah Lake. The final saline population was collected on the eastern edge of the Great Salt Lake

Desert, the dry lakebed remnant from the Pleistocene era Lake Bonneville. The 5 nonsaline populations were collected on mountainsides spread throughout Salt Lake Valley and Utah Valley. The two closest populations are 3 km apart, while the two furthest are 120 km apart. While there could be some concern over gene flow between populations close to each other, prior work shows very little gene flow between *Bromus* populations, even on small scales, due to rare outcrossing events (Novak et al. 1991, Schachner et al. 2008). I, therefore, assume that each of these populations serves as an independent replicate of populations associated with saline or nonsaline environments.

At the same time that I collected the seed material (June 2019), I also collected soil material at each population to provide inoculum for subsequent greenhouse experiments. I collected soil from the top 15 cm of soil throughout 1 square meter at the center of the population using a sterilized trowel. Soils were placed in a plastic bag, returned to the lab, and then stored at 5°C until use in subsequent greenhouse experiments.

From each population, I collected seeds from 10 separate plants, from which I started 10 maternal family lines in a greenhouse environment. Seed was collected from randomly selected plants distributed across approximately 10 square meters. These plants were grown for a single generation in the greenhouse in order to equilibrate maternal effects across populations and produce seeds for subsequent experiments. Briefly, seeds were vernalized for 8 weeks at 5°C to facilitate flowering (Meyer et al. 2004), after which they were sown in small "cone-tainer" pots and filled with approximately 120 mL of a locally produced "root-wash" soil mix: equal parts of calcined clay, torpedo sand, and field soil (University of Illinois Plant Care Facility, https://pcf.aces.illinois.edu/soil-mixes/). Plants were grown in the greenhouse on a 26°C/24°C day/night schedule and supplemented with 14 h of daily light. Plants began to produce seeds

between 12 to 20 weeks after sowing. Upon seed ripening, seeds were collected and incubated at 30°C for an additional 6 weeks to facilitate after-ripening (Christensen et al. 1996). *Bromus tectorum* is cleistogamous (self-pollinating) with minimal out-crossing (Novak and Mack 1993) and therefore the seeds collected from each maternal line were likely full-sibs, minimizing genetic variation within families. In total, I generated 100 maternal family lines, with 10 lines per population.

Greenhouse experiment

I assessed the degree to which each family line was adapted to saline and nonsaline soil conditions, as well as the degree to which microbial interactions influenced this adaptation in a greenhouse-based reciprocal transplant experiment. In July 2020, I grew all *Bromus* lines in the greenhouse under both saline and nonsaline conditions. I also crossed the salinity treatment with 3 microbe treatments: live microbes using soil inoculum collected from nonsaline populations, live microbes using soil inoculum collected from saline populations, and sterile inoculum. While I designed this experiment with a key comparison being between live and sterile conditions, by including live inocula from both saline and nonsaline habitats, our experiment additionally included a *reciprocal microbe transplant component*, which was orthogonal to the *Bromus* transplant treatment. This design provided two *a priori* contrasts allowing us to assess 1) whether there is any microbial role in plant adaptation to salinity by comparing live microbe treatments to sterile soil treatments and 2) whether "salinity-matched" plant-microbe interactions were necessary for salinity adaptation by determining if plants paired with microbes that came from their "matched" salinity environments (e.g., saline plants with saline microbes) responded differently to saline vs. nonsaline environments relative to plants with "mismatched" microbes

(e.g., saline plants with nonsaline microbes). This secondary comparison allows us to evaluate whether there are specific microbes that may be facilitating plant adaptation.

I created two live microbial treatments, inoculum from saline habitats and inoculum from nonsaline habitats: these were composite inoculums generated by mixing equal parts of the soils from the 5 populations of the respective salinity habitat. Soils used for the inoculum had been stored at 5°C since collection approximately 12 months earlier. In combination with our sterilized inoculum, this resulted in three distinct microbial treatments, which I treated as a fixed effects factor in our experimental design. The pooling of inoculum does limit the inference scope, as I homogenized site-to-site variability of microbial salinity responses even while I retained replication across plant lines and populations. In particular, we cannot estimate the variance of microbial effects across sites (just across plant lines and plant populations), and I cannot estimate the effects of salinity on microbes. While I would have ideally instead included 10 live inoculum treatments, corresponding with the soils from each of the 10 *Bromus* populations, this would have expanded the experiment beyond the limits of our capacity. Instead, I constructed microbial inocula that were intended to represent microbes that would be found at the two extreme ends of the salinity gradient.

I grew our 100 maternal lines of *Bromus* in the greenhouse, on a 26°/24°C day/night schedule, supplemented with 14 h of daily light. Plants were grown in cone-tainer pots and filled with approximately 120 mL of a root-wash mix (see the previous description for details of the soil mix). I sterilized soils by autoclaving (3 times in 1 h cycles, with 20 min. rests between cycles) shortly before the experiment. I then amended soil 10% by volume with the designated soil inoculum, mixing soils using a sterilized cement mixer. Plants assigned to the sterile treatment received sterilized inoculum at identical volumes to the live treatments (a 1:1 mix of

the saline and nonsaline inocula), to control for potential chemical/nutritional differences between treatments. This sterile inoculum was autoclaved and mixed into the designated soils identically as above. For every treatment combination of population, maternal line, salinity, and microbes, there were 3 replicates, for a total of 1800 plants (10 populations x 10 maternal lines/population x 2 salinity treatments x 3 microbe treatments x 3 replicates).

Prior to starting our greenhouse experiment, I surface-sterilized seeds by immersing seeds in a 2% sodium hypochlorite and 0.05% Tween 80 solution and vigorously stirring for 2 minutes, followed by a thorough rinsing in sterile water. I then placed seeds onto sterilized moistened filter paper to allow germination. I placed 3 germinated seeds into each pot, covering them with a thin layer of soil. I randomized the position of pots on the greenhouse bench to minimize environmental variation, and regularly rotated their position on the bench. I thinned pots to 1 individual/pot in the subsequent weeks. I watered pots every 3 days to saturation to maintain soil moisture. A small portion of the pots across all populations did not have any successful seedlings emerge in the first few weeks (~13% of all pots; Figure A.3). I excluded these failed plants from all subsequent analyses.

Four weeks after planting seeds, I began salinity treatments. I continued to water plants assigned to the nonsaline treatment every 3 days to saturation. On the same watering schedule, I watered plants assigned to the salinity treatment with a 0.6% NaCl solution for 1 week. After 1 week, I then began watering these same plants instead with a 1.2% NaCl solution for the remainder of the experiment. When watering, I flushed pots with either freshwater or the saline solution (based on their assigned salinity treatment) to minimize the buildup of salts in the soil over the course of the greenhouse experiment. I did not initially start our plants under saline conditions and included this short ramping of soil salinity primarily to avoid stress shocking the

plants. Moreover, in this region, early spring is the wet season with frequent rainfall; as the season progresses, rainfall is less frequent and consequently osmotic pressure in these saline playa populations increases throughout the season as soil water slowly evaporates, leaving behind residual salts and causing increasing osmotic stress (Scott et al. 2010). Including this ramping of soil salinity thus somewhat mimics natural conditions experienced by the saline populations. I chose the 1.2% NaCl solution as the final concentration for the salinity treatment as this concentration set soil conductivities at ~6 dS/m, which a pilot experiment demonstrated that this concentration imposed significant plant stress without resulting in 100% mortality, while also being on the low range of the conductivities observed in the soils of the saline populations (Figure A.2). Thus, I created two greenhouse salinity environments that were reflective of these populations' likely home salinity environments. I acknowledge that this experimental design ignores the underlying variation in salinity conditions amongst these habitats, while also ignoring other abiotic or biotic components that may be important to their adaptation. Indeed, by narrowing our test of adaptation to a single abiotic variable, it can be viewed as a conservative test of the microbial impact on plant local adaptation, with significant results potentially suggesting that I captured relevant variation.

I recorded the dates of plants' death throughout the experiment to assess population survival. Ten weeks after the initiation of the experiment, I harvested above- and belowground biomass over the course of 5 days. This timing represented 6 weeks of applying salinity stress out of the total 10 weeks of growth. As our pilot studies showed that some populations of *Bromus* completed their life cycle under greenhouse conditions in as few as 12 weeks, this length of experiment represented a significant portion of the plant's generation time. After harvest, I

gently washed root tissues to remove soil particles. All tissues were oven-dried at 75°C for 72 h and then weighed.

Data analysis

I used total biomass (composite of above- and belowground biomass) and survival data as measures of plant adaptation to our various greenhouse treatments. I assumed that increasing biomass and survival for a given habitat represents increased fitness of the plant to that environment as prior work has shown a strong correlation between *Bromus* reproductive fitness and aboveground biomass (Leger et al. 2014, Smull et al. 2019). While belowground biomass is not frequently used as a proxy of plant fitness, some have suggested measuring multiple traits related to plant health to fully encapsulate plant fitness (Mason et al. 2017, Younginger et al. 2017). Moreover, a prior study examining adaptation in *Bromus tectorum* to saline habitats found the largest evidence for adaptation in changes in root biomass (Scott et al. 2010). I did not measure reproductive fitness as *Bromus* requires extensive periods of vernalization for flowering (Meyer et al. 2004), which in our pilots resulted in low germination rates (I speculated this may have been due to mold issues). As I was limited in the number of seeds, I chose not to risk losing all our seeds in a failed vernalization treatment.

Given a significant number of our plants under the saline greenhouse treatments died due to the stressful environment, these plants had effectively zero fitness. I did not want to exclude these plants from the analysis as they represent an important component of adaptation, however, the large number of zeros made model construction difficult. I consequently used the aster model approach of Geyer and colleagues (2007) to create a single composite estimate of fitness, which unified biomass measures and survival data with their appropriate statistical distribution. I

modeled biomass and survival with normal and Bernoulli distributions respectively. From here on, I will refer to this aster model as our estimated fitness; while none of the traits input into these models are a direct measure of fitness, they are effective proxies (see above).

In our aster model, I included as fixed effects: a plant's historical salinity environment (saline or nonsaline), greenhouse salinity environment (saline or nonsaline), microbe inoculum treatment (saline microbes, nonsaline microbes, or sterile), and the interaction of these terms. To control for non-independence among individuals from the same population, I additionally included population (nested within historical habitat) as a random effect, as suggested by Kawecki and Ebert (2004). While I attempted to include maternal line as a random effect, this failed as estimating the variance components for both all the population and maternal line effects in aster models was too demanding of the data. Given the maternal lines within each population were sampled fairly close to each other, they may have been close relatives. Therefore, population may statistically be the most appropriate level of sampling to consider. As a check on our work though, I do additionally include in the supplemental the same models but with only maternal line as a random effect (Table A.2; I note little difference in these models). I determined the significance of the fixed effects by using likelihood ratio tests, comparing sequentially nested models with and without the term of interest. I constructed this model using the aster package in the R environment (Geyer et al. 2007, Geyer 2021).

Given that the estimated fitness using the aster model was a composite of both survival and the component biomass (above- and belowground biomass), I additionally examined these individual components in a similar analysis as above to identify how each of these contributed to salinity adaptation. Specifically, I constructed similar models as those described above for survival, and above- and belowground biomass. For biomass, I specifically examined biomass

from plants that had not died, as this allowed us to examine whether adaptation was driven by changes in survival and/or biomass. Given survival is a binary outcome, I accordingly analyzed survival by constructing a generalized linear mixed effects model with a binomial error distribution. Given survival for plants under the nonsaline treatment was unsurprisingly near 100%, I exclude that treatment from the analysis. For both above- and belowground biomass, I squareroot-transformed biomass to meet assumptions of normality and constructed mixed-effects models using the lme4 and lmerTest packages (Bates et al. 2015, Kuznetsova et al. 2015).

Local adaptation to different environments is often evaluated in reciprocal transplant experiments by examining the two-way interaction between an organism's source habitat, and the contemporary habitat; in this case the source habitat and the greenhouse salinity treatment (Blanquart et al. 2013). However, to determine if plant-microbe interactions are important for an adaptive salinity response, I focused on the three-way interaction between the historical salinity environment, greenhouse salinity, and inoculum as this term would act as an indicator of the role of microbes in influencing adaptation. To further evaluate the sign and the relative impacts of treatments, I used specific contrasts associated with these hypotheses that were chosen *a priori*, allowing for more powerful analyses than *post hoc* comparisons. Namely, within each combination of inoculum and greenhouse salinity, I compared our estimated fitness measure (aster model, biomass, and survival) between the 2 historical source salinity habitats (see Figure 2.1 for predictions and planned contrasts; contrasts are between genotypes within each environment). Salinity adaptation would be supported if, for a given greenhouse salinity treatment, I observed the highest fitness in plants whose historical salinity environment matched the greenhouse salinity treatment (e.g., saline plants with saline treatment, and nonsaline plants

with nonsaline treatment). I could then evaluate the importance of microbes to salinity adaptation by comparing patterns of adaptation with these comparisons within each microbial treatment.

While the primary goal of this contribution was to isolate the interactive biotic and abiotic components of plant adaptation to their home salinity environments, I additionally examined whether the identity of the microbial inoculum influenced these patterns of adaptation. To this end, I made additional comparisons with these estimated fitness measures, comparing the impact of matching a plant with its matched inoculum (e.g., saline genotypes with saline inoculum) vs. the mismatched inoculum (e.g., saline genotypes with nonsaline inoculum). The inclusion of the sterile microbe treatment was not required for these comparisons.

RESULTS

The 3-way interaction between habitat, greenhouse salinity, and inoculum significantly influenced our estimated fitness (aster model using total biomass and survival, Table 2.1: $p < 0.001$, see also Table A.2). This significant interaction term suggested that any adaptation to the home salinity environment was significantly influenced by the microbial treatment; however, from this interaction alone, it is not clear *how* microbes may be influencing adaptation. I therefore used pairwise comparisons to parse this interaction, as described below.

Based on the estimated fitness aster model, our pairwise comparisons suggested that plants from saline habitats were better adapted to high-salinity soils than plants from nonsaline habitats, but only when they were grown with live microbes (Figure 2.2). Under saline conditions, saline genotypes had significantly higher relative fitness than nonsaline genotypes when paired with a live microbial inoculum (Figure 2.2A, 2.2B, Table A.3). However, when

grown with a sterile inoculum, saline genotypes did not have statistically different fitness estimates than nonsaline genotypes (Figure 2.2C).

Similarly, our pairwise comparisons suggested that plants from nonsaline habitats were better adapted to nonsaline soils than plants from saline habitats, but only when grown with live microbes (Figure 2.2). Under nonsaline conditions, nonsaline plants had significantly higher relative fitness than saline genotypes when paired with the saline inoculum, though not with the nonsaline inoculum (Figure 2.2A, 2.2B, Table A.3). However, when grown with a sterile inoculum, nonsaline genotypes did not have statistically different fitness estimates than saline genotypes (Figure 2.2C).

Similar patterns of salinity-matching in the presence of microbes could be found in both the survival and the component biomass data. For example, under saline conditions, saline genotypes had higher survival rates, and both above- and belowground than nonsaline genotypes, however only when paired with a live microbial inoculum (Figure A.4 and A.5, Table A.3). Under nonsaline conditions, nonsaline genotypes had significantly higher belowground, but not aboveground biomass, than saline genotypes, however only when paired with a live microbial inoculum.

I found minimal effect of matching a plant with its salinity "matched" inoculum on our estimated fitness aster measure (e.g., saline genotypes with saline inoculum; Figure 2.3). In one case, I found that nonsaline genotypes had significantly higher estimated fitness when paired with their mismatched microbes (nonsaline microbes), however this was only when they were in a foreign salinity environment (saline). Conversely, I found that saline genotypes had significantly lower estimated fitness when paired with their matched microbes (saline microbes)

than with when paired with their mismatched microbes (nonsaline microbes) while under saline conditions. All other comparisons of had nonsignificant impacts on estimated plant fitness.

DISCUSSION

Overall, our results suggest that the apparent adaptation of *Bromus tectorum* populations to their historical salinity environment was significantly influenced by interactions with soil microbes. Specifically, under saline conditions with any live inoculum, *Bromus* genotypes from saline habitats had higher biomass and survival than genotypes from nonsaline habitats, while under nonsaline conditions with any live inoculum, genotypes from nonsaline habitats had higher biomass than genotypes from saline habitats. This result is consistent with the expectation that populations should be locally adapted to their home salinity environment (Figure 2.1A; i.e., higher relative fitness in their home habitat than foreign genotypes). However, under sterile soil conditions, these patterns disappeared. Moreover, while the fitness of nonsaline plants and saline plants did not statistically differ across salinity treatments under both saline and nonsaline environments in these sterile soils, plants appeared maladapted to their home salinity conditions, with plants trending towards having higher relative fitness estimates and biomass in their foreign salinity environment (Figure 2.2C, Figure A.5C and A.5F). Taken together, these results suggest that the observed patterns of plant salinity adaptation may have been partially influenced by plant interactions with soil microbes. However, on average, plants had higher fitness in sterile soils than in soils with live microbes, which may imply that microbial interactions may have been a net negative for *Bromus* and significant drivers of the fitness patterns.

Through what mechanisms are these plant-microbe interactions influencing plant salinity adaptation? Prior work in resource mutualisms such as rhizobia and mycorrhizal symbioses has

shown that plants may have the highest fitness when paired with microbial genotypes that have a shared evolutionary history (Johnson et al. 2010, Batstone et al. 2020a). In adapting to specifically their biotic environment, such coevolutionary dynamics between plants and their associated microbes could generate patterns expected in local adaptation (Figure 2.1A). While this experiment was not necessarily designed to address this hypothesis, I can *post hoc* examine if there is support for this hypothesis by comparing plant fitness across the two microbial inocula. For plant-microbe coevolution to drive salinity adaptation, I should have found plants having the highest relative fitness in their home salinity environment when grown with microbial inoculum that was matched to the historic salinity environment, where plant and microbial genotypes would have been most likely to share an evolutionary history. However, there is no support that these coevolutionary dynamics facilitated the observed microbial benefit in these plants' home salinity environment, as overall "salinity matched" inocula were not better than "mismatched" inocula (Figure 2.3). In one case, there was a small positive benefit to plant fitness when matching nonsaline plants with their home inoculum, however, this was in a salinity treatment foreign to the plant. In another case, saline plants were more fit in foreign inoculum which could be due to the release from one's own specialized pathogens (i.e., enemy release hypothesis; Liu and Stiling 2006) or pathogens from saline environments being adapted to saline conditions. It may be the case that our pooling of soil inocula from multiple sites with the same salinity conditions could have obscured any signal of coevolution by disrupting particular plant-microbe interactions between site-specific combinations of plants and microbes. However, given that I observed patterns of plant salinity adaptation across both live microbial treatments and our inability to detect a "salinity matching" effect, this likely indicates that the fitness patterns I

found here resulted from generalized plant interactions with microbes functionally redundant across both live microbial communities.

I, therefore, speculate that these patterns of the salinity adaptation in *Bromus* may be generated by plant immune responses adapted to their home salinity environments to broadly available antagonistic/pathogenic microbial activities. I have noted that plants grown under sterile conditions on average had higher survival and biomass than plants inoculated with live soils, suggesting that the soil may harbor microbes that are detrimental to *Bromus* or that *Bromus* is particularly susceptible to pathogen pressure. Moreover, negative microbial effects were always more severe when plants were grown in their nonnative salinity environment, while release from microbial interactions in sterile soil resulted in similar fitness measures between saline and nonsaline-adapted populations. I suggest that the observed patterns of *Bromus* local adaptation may be driven by the fact that plant fitness may be co-limited by salinity and pathogen pressure. While it may seem counterintuitive that net negative microbial effects could cause a plant population to appear to be better adapted to its home salinity environment, these plants have evolved in the presence of microbes (versus under sterile conditions), so plant genotypes that had less detrimental interactions with the soil microbiota would have a competitive advantage that could be selected on, even if microbial interactions were still overall negative. For example, in foreign environments an organism's immune system may be weakened and less able to fend off pathogenic microbes compared to when it is in its home environment (Karl et al. 2010), exacerbating the stress of the foreign environment (David et al. 2018). Consequently, if there are widely distributed generalist pathogens of *Bromus*, plants may appear locally adapted to their home salinity environment as a result of immune systems optimized to these salinities. Similarly, given there may also be overlapping molecular mechanisms in plants

responsible for both tolerance to osmotic stress and pathogens (Asselbergh et al. 2008, Ranty et al. 2016), plant adaptation for tolerance to pathogens or saline environments may incidentally facilitate adaptation to the other. Overall, under this pathogen-driven hypothesis, microbes are not necessarily mediating adaptation to these salinity environments. Rather, their presence alters the relative fitness difference of plants in a comparison of local vs. transplanted genotypes that produce patterns identical to what is expected for salinity adaptation.

There have unfortunately been few studies investigating the *Bromus* rhizosphere communities, so it is difficult to determine whether pathogenic interactions are common in this species and therefore likely drivers of our observed patterns of fitness. While there have been several specialized pathogens identified associated with *Bromus* (Meyer et al. 2016) this is not necessarily indicative of it being particularly susceptible to pathogens as these were identified for the potential application as a biocontrol of invasive *Bromus*. Future work may therefore attempt to connect specific plant-associated microbial communities with their impact on plant adaptation.

While I have thus far focused on how negative and pathogenic interactions may be driving these patterns of plant adaptation, I acknowledge that these patterns could alternatively be the result of beneficial microbial interactions lurking in this net-negative effect. These hidden beneficial interactions could underly the reduced antagonism I found when plants were grown in their home salinity condition. Such hidden beneficial interactions could emerge as the product of selection on plant heritable plant traits that facilitate beneficial interactions under a high- or low-salinity condition (Aira et al. 2010, Gehring et al. 2017, Walters et al. 2018, Bergelson et al. 2019).

Without detailed microbiome-level descriptions of microbial communities, it is not possible to pinpoint specific microbial taxa, whether with positive or negative impacts on the

plant, that may be influencing our measures of plant fitness or how these contribute to patterns of plant salinity adaptation. I have estimated the net effects of the microbial inocula in our experiment, and a more detailed investigation of the resultant microbiomes could identify species-specific effects. Similarly, without plant genomic data, I cannot pinpoint particular plant genes responsible for these effects; even with such genomic data though, it would likely be difficult to identify potentially relevant genes as there would likely be a suite of genomic changes in these populations not associated with the plant's microbial interactions. This level of detail for both plant genomics and soil microbiome is outside the scope of this study. Nevertheless, the broad patterns of plant responses across the salinity and microbial conditions do suggest several directions for future mechanism-focused research.

I briefly note that there is significant variation in soil conductivity between our saline populations (Figure A.2). If these plants are adapting to saline environments, I might expect those populations from the higher conductivities to have the highest fitness. While under saline greenhouse conditions and with live microbes, there broadly was higher fitness in the saline-adapted populations compared to the nonsaline, variation in these saline populations' fitness was not related to their home soil conductivity (Figure A.6). This may be a result of sampling design, as I only had 5 populations from the saline habitats, limiting inference.

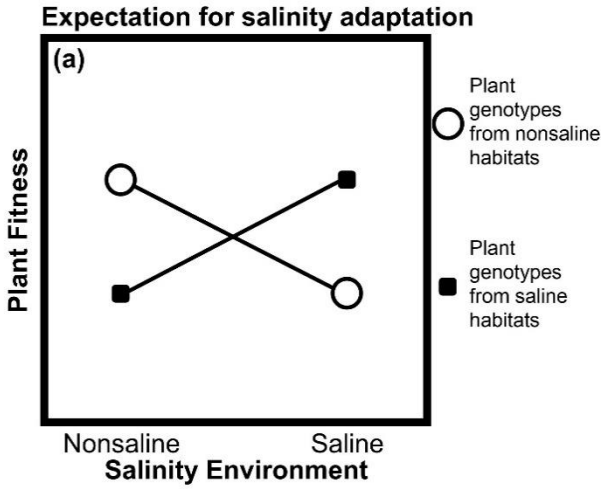
CONCLUSION

This work highlights that host-microbe interactions may be key in shaping the patterns of plant adaptation. Similarly, several recent studies have provided results similar to our own, wherein host populations were only locally adapted to their home environment when paired with live microbes (Gehring et al. 2017, Macke et al. 2017, Henry et al. 2020). While these results cannot

point to an exact mechanism, they, as well as those of others, emphasize the significant impact of microbes on their host's fitness, likely acting as a significant selective agent on their host, and overall suggesting that these host-microbe interactions may play a role in shaping the observed patterns of host adaptation to their local environment. Indeed, many traits long associated with the host, including development, morphology, physiology, etc. are being linked with the host's associated microbes (Friesen et al. 2011, Theis et al. 2016, Haag 2018). Host-microbe interactions may be altering host evolutionary potential (Henry et al. 2021) by acting as significant targets for natural selection and as the traits underlying host adaptation. I call for further integrating the microbial component into the host's traits and selective environment as such work will lead to further insights into the drivers of the evolution of host-microbe symbioses as well as their adaptation.

FIGURES AND TABLE

Figure 2.1: I display the expectations for patterns of plant fitness when adapted to its local environment (salinity in this case). I additionally display how adaptation may be influenced by the plant's interactions with its associated microbes.



Hypothesis: Given a plant's associated microbes may be strong agents of selection on their host through their impact on plant fitness, patterns of plant adaptation may be dependent on interactions with their associated microbes

Prediction: Plants will be adapted to home environment only when provided with live microbes

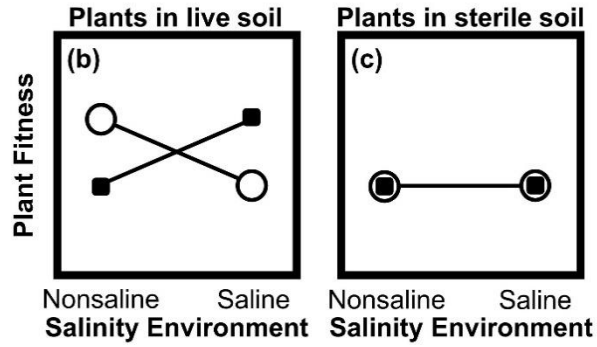


Figure 2.2: Estimated fitness from our greenhouse experiment, using aster models to combine total biomass (mg) and survival data into one composite fitness measure. Data are grouped by the plant's habitat of origin (saline and nonsaline populations), greenhouse salinity treatment (saline and nonsaline), as well as the inoculum provided (saline microbes, nonsaline microbes, and sterile). I display both the data for individual populations, as well the average of populations based on their habitat of origin. Individual populations are semi-transparent, while averages of habitat are solid. I chose specific orthogonal contrasts *a priori* to evaluate salinity adaptation; namely, I compare the habitat of origin under each salinity and microbial combination, as indicated in the figure, as is appropriate to evaluate local adaptation. Bars represent 95% confidence intervals of the mean generated from the standard error. The statistical significance of each comparison is indicated using the symbology as follows: ns $p > 0.10$; + $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

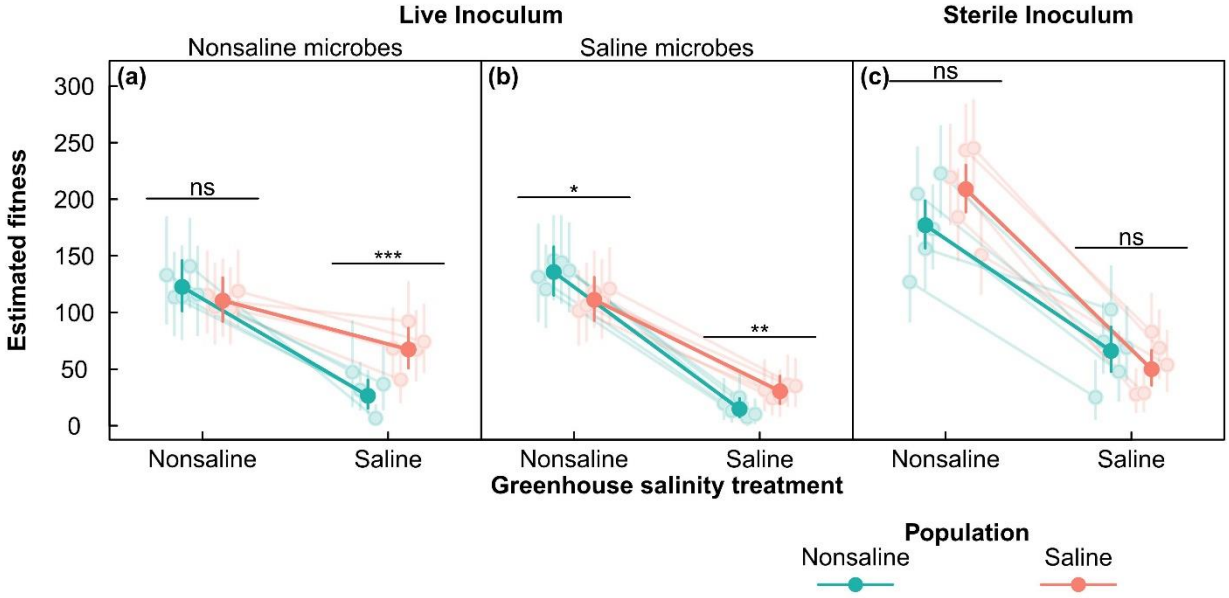


Figure 2.3: Estimated fitness from our greenhouse experiment, using aster models to combine total biomass (mg) and survival data into one composite fitness measure. Data are grouped by the plant's habitat of origin (saline and nonsaline populations), greenhouse salinity treatment (saline and nonsaline), as well as the inoculum provided (saline microbes and nonsaline microbes). I note that this figure utilizes the same data from Figure 2.2, except that the panels display different treatment pairs, and the sterile treatment is excluded. Specifically, I wanted to assess the impact of matching a plant population with its home inoculum. For example, for saline populations the saline microbes are the home inoculum while for the nonsaline populations the nonsaline microbes are the home inoculum. I display both the data for individual populations, as well as the average of populations based on their habitat of origin. Individual populations are semi-transparent, while averages of habitat are solid. Bars represent 95% confidence intervals of the mean generated from the standard error. The statistical significance of each comparison is indicated using the symbology as follows: ns $p > 0.10$; + $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

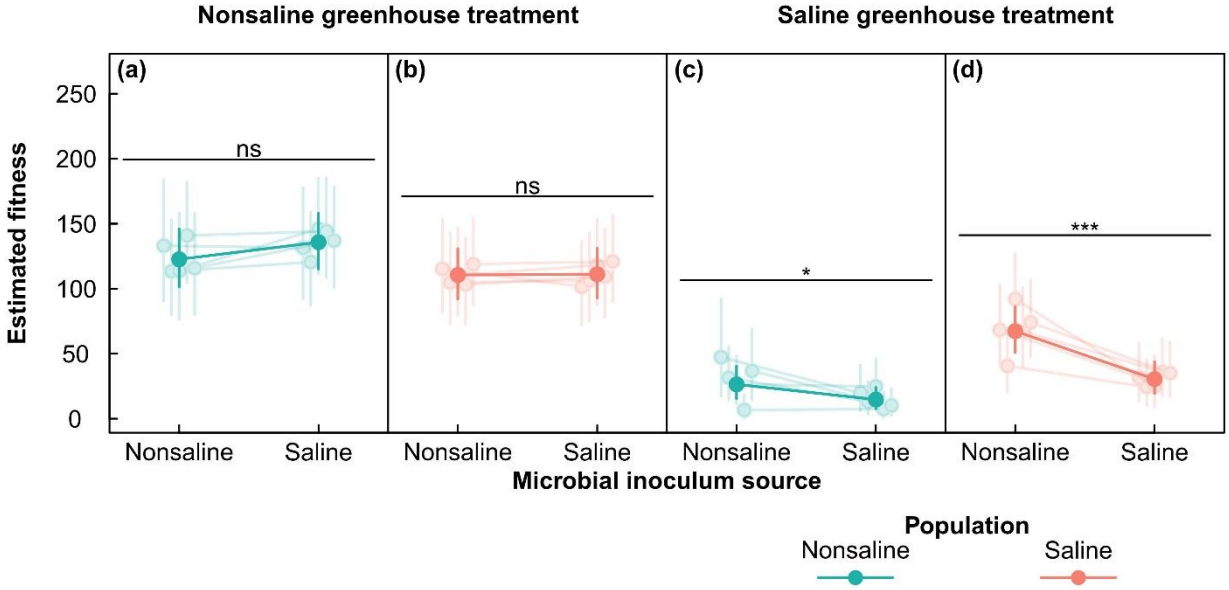


Table 2.1: Model evaluating the effects of the plant's habitat of origin (saline and nonsaline populations), greenhouse salinity treatment (saline and nonsaline), the inoculum provided (saline microbes, nonsaline microbes, and sterile) and their interactions, on the estimated fitness (total biomass and survival, using aster models), with population as a random effect. Analogous model using maternal line as a random effect can be seen in Table A.2. Separate models were also built for the components going into this measure of estimated fitness, including above- and belowground biomass, and survival. For survival, terms including the salinity treatment were excluded, as there was no variance in survival under nonsaline treatments (near 100% survival). For estimated fitness, I display the deviance from the aster model for each model term. For above- and belowground biomass models I display both the F value and Mean Square for each model term. For survival, I display the Chi-Squared for each model term. Terms with an associated *p* value less than 0.05 are bolded. + $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Model Term	**df**	**Estimated Fitness (Total Biomass + Survival)**	**Aboveground**		**Belowground**		**Survival**
		Deviance	MS	F value	MS	F value	ChiSq
Habitat Source	1	2.2085	1.47	0.35	10.5	1.53	2.23
Salinity	1	**500.08*****	**160.22**	**37.55*****	**3943.4**	**579.05*****	NA
Inoculum	2	**102.28*****	**65.47**	**15.35*****	**1312.6**	**192.73*****	**13.85*****
Habitat x Salinity	1	**7.2065****	**28.87**	**6.76****	**99.5**	**14.62*****	NA
Habitat x Inoculum	2	4.9821^{+}	**22.39**	**5.25****	17.3	2.54^{+}	**11.27****
Salinity x Inoculum	2	**38.591*****	**67.06**	**15.72*****	**215.5**	**31.64*****	NA
Habitat x Inoculum x Salinity	2	**28.624*****	**26.25**	**6.15****	**175.1**	**25.72*****	NA

CHAPTER 3: SOIL MOISTURE INCIDENTALLY SELECTS FOR MICROBES THAT FACILITATE LOCALLY ADAPTIVE PLANT RESPONSE

ABSTRACT

While a plant's microbiome can facilitate adaptive phenotypes, the plant's role in selecting for these microbes is unclear. Do plants actively recruit microbes beneficial to their current environment, or are beneficial microbes only an incidental byproduct of microbial adaptation? I addressed these questions through a multigeneration greenhouse experiment, selecting for either dry- or wet-adapted soil microbial communities, either with or without plants. After 3 plant generations, I conducted a full reciprocal transplant of each soil community onto wet- and dry-treated plants. I found that plants generally benefited from soil microbes, and this benefit was greater whenever their current watering conditions matched the microbes' historical watering conditions. Principally, the plant's presence was not necessary in the historical treatments for this environmental matching benefit to emerge. Moreover, I found microbes from droughted soils could better tolerate drought stress. Taken together, these results suggest that the moisture environment selects for microbes that benefit plants under those specific moisture conditions, and that these beneficial properties arise as a byproduct of microbial adaptation to the watering environment and not as a co-adapting plant-microbe system. This work highlights that understanding the selective agents on these plant-associated microbes will lead to a better understanding of plant adaptation.

INTRODUCTION

The degree to which a plant's phenotype is adapted to its environment is often considered a product of the plant's evolutionary history and genetics (Hoban et al. 2016, Tiffin and Ross-Ibarra 2017). However, a plant's phenotype is plastic, in part due to the composition and function of its associated microorganisms (Vandenkoornhuyse et al. 2015, Theis et al. 2016). Plants are colonized by diverse communities of microorganisms that can influence plant phenotype through their various activities including but not limited to, nutrient cycling, decomposition, hormone production, and pathogen activity (Falkowski et al. 2008, Van Der Heijden et al. 2008, Martin et al. 2017). While these plant-microbe interactions are key in determining the fitness of both the plant and microbial partners, the evolutionary history and selective agents behind many of the microbial traits that influence plant phenotype are unclear. Identifying the selective pressures on plants' associated microbes, and how this selection influences their impacts on host adaptation will provide insight to these microbes' operation and evolution, as well as their contribution to plant phenotype. To this end, herein I investigated the selective agents on plant-associated microbes that determine these microbes' impact on plant adaptation to different soil moisture environments, specifically focusing on the separate contribution of the host plant and the abiotic environment in selecting for microbes that mediate locally adaptive plant phenotypes.

Recent work has highlighted that plant hosts are frequently colonized by microbial communities that mediate locally adaptive plant phenotypes (Millar and Bennett 2016). For example, prior work has found that plants in a drought experiment had the highest fitness when provided with microbes collected from plants whose wet/dry-watering environment matched the plants' current watering environment (Lau and Lennon 2012, Giauque et al. 2018, O'Brien et al. 2018, Allsup and Lankau 2019), and similar results have been found in linking microbes to plant

adaptation to serpentine soils and across temperature and salinity gradients (Redman et al. 2002, Doubková et al. 2012, Yuan et al. 2019). These patterns may indicate that plants and microbes adapt to environmental stress as a co-evolving system, which implies that the plant acts as a significant agent in selecting for microbes that facilitate its locally adaptive phenotype.

Indeed, through their root morphology, chemistry, root exudation patterns, immune signaling, etc., plant roots select upon their root-associated microbes, provisioning important resources and habitats for many soil microbes and influencing which microbes can colonize the plant root (Broeckling et al. 2008, Berg and Smalla 2009, Zhalnina et al. 2018, Trivedi et al. 2020, Williams and de Vries 2020). Prior work has hypothesized that plants may have evolved mechanisms to preferentially recruit microbes that provide an adaptive value to the plant; namely, that plants use a 'cry for help' in response to various environments, wherein plants exposed to stress adaptively alter their root exudates and chemistries to recruit microbes beneficial to the current environment (Rolfe et al. 2019, Liu et al. 2021, Rizaludin et al. 2021). Similarly, the microbial dependence on the plant for resources may select from microbes with cooperative traits that support locally adaptive plant phenotypes that maintain plant health and thus resources for the microbes (Li et al. 2021). The mutual dependence of plants and microbes could potentially align plant and microbial fitness in the face of environmental stress. Therefore, there may be selection for microbes that facilitate locally adaptive plant phenotypes, because the microbial traits that increase plant fitness will also consequently benefit microbial fitness.

However, the plant is not the sole selective agent on their associated microbes. Many root-associated microbes are not fully obligate on the plant and can also be free-living in the soil (Lagos et al. 2015). Consequently, these microbes must navigate multiple dimensions of selection besides the plant -- especially environmental stress. Any microbial traits that benefit the

plant in a given environment may thus alternatively be the result of microbial adaptation to that environment, with any benefit to the plant only an incidental byproduct. For example, traits that help microbes persist in the face of environmental stress will directly benefit their own fitness under similar environmental conditions. However, by maintaining important microbial functions under environmental stress, these microbial traits may also end up benefitting plants. Importantly, these beneficial traits would be an incidental byproduct of selection on these microbes from the local environment.

To understand the origin of microbial interactions beneficial to the plants in their local environment, I compared the contribution of the host plant and the abiotic environment in selecting for microbes that facilitate locally adaptive benefits to the plant. In addressing these questions, I used a long-term greenhouse experiment, exposing soil microbial communities to various selective watering environments (wet vs. dry) for multiple plant generations. Following these selective treatments, I used a full reciprocal cross and inoculated these microbial communities back onto plants under the two original watering treatments, allowing us to evaluate these microbes' contribution to plant adaptation to the historical watering treatments. To evaluate the importance of plant selection on microbes, I included two plant conditioning species. Additionally, I critically included a "no plant" control treatment to determine if plants and microbes need to adapt to drought stress *together*. By comparing the fitness of plants inoculated with microbes from "plant" and "no plant" treatments I can identify the importance of the plant versus the environment in mediating selection on the microbes, and the subsequent contribution to plant phenotypes adaptive to moisture environment.

MATERIALS AND METHODS

Greenhouse experiment

Selection Phase (Generations 1–3)

I established a long-term greenhouse selection experiment to investigate selective agents on plant-associated microbes and their impact on plant adaptation. For three plant generations, I exposed soil microbes to two selective moisture treatments, wet or dry. I refer to this treatment as the historic moisture selection treatment. I crossed this moisture treatment with three plant treatments: 1) *Brassica rapa* (standard stock lines, Wisconsin Fast Plants Program, University of Wisconsin); 2) *Arabidopsis thaliana (*Col-0 lines, Arabidopsis Biological Resource Center, Columbus OH, USA), and 3) no plants present. I refer to this treatment as the historical plant selection treatment. Altering the presence of plants in this experiment altered the presence of plant selection on the microbes, and including the two separate plant species allowed us to evaluate the importance of species-specific specialization of soil microbes to the selective plant.

In the first generation, soil microbial communities were established in small "cone-tainer" pots and filled with approximately 120 mL of a locally produced "root-wash" soil mix: equal parts of calcined clay, torpedo sand, and field soil (University of Illinois Plant Care Facility, https://pcf.aces.illinois.edu/soil-mixes/). To provide a microbial community that could be the target of selection by the various selective treatments, soil was amended 5% by volume with a live soil inoculum field-collected from a decades old tallgrass prairie restoration site (40.128577, -88.140734). There were 30 replicate pots in each treatment, for a total of 180 pots (30 reps x 2 watering treatments x 3 plant treatments). At the start of each of the first three generations, I planted 5 seeds of the designated species in each pot. All pots were watered to saturation daily for the first week after sowing to facilitate successful germination, after which I

thinned pots down to 3 plants and the moisture treatments were imposed: in the wet treatment, pots were watered to saturation every 2 days; in the dry treatments, I weighed pots and watered to 14% gravimetric water content every 2 days. I chose this water content target for the dry treatment because a pilot study demonstrated this water content represented a significant stress compared to the well-watered control, while still allowing for plant survival. These watering treatments thus allow for differing selective environments, which can be used to evaluate plant adaptation to each moisture environment. Plants were grown in the greenhouse on a 26°C/24°C day/night schedule, supplemented with 14 h of daily light. I continued these treatments for a total of 3 plant generations, with each generation lasting approximately 50 days. Between each generation, plant shoots were pruned, and new seeds of the appropriate species were sown into pots to continue the plant selection treatments.

Test Phase (Generation 4)

After 3 generations of selection on the soil microbial communities, I examined how the resulting microbial communities mediated the plant's response to the selective moisture environments. To this end, I performed a full reciprocal transplant on the soil from all pots, using soils from each pot as inoculum for a new generation of plants. To control for potential nutritional/chemical changes in soils due to the historic selective treatments, the soils from each pot were divided into two, with one portion being autoclaved. Both live and sterile (autoclaved) components were used as separate sources of inoculum into freshly sterilized soil in this final generation. Comparing subsequent plant growth between paired plants inoculated with the live soils and those inoculated with sterile soils would allow us to compare the microbial impact of these historic selection treatments apart from the nutritional/chemical changes to the soil. This approach is similar in

design to plant-soil feedback experiments (Brinkman et al. 2010). I freeze-dried 5 g soil inoculum from each of these pots to be used for nutrient analysis. Additionally, for future microbial analyses, I stored 1 gram of soil from each mesocosm in a 10 mL solution of 70% PBS and 30% glycerol and stored at -80°C.

Each soil inoculum was inoculated into 2 pots, with each assigned to either a wet or a dry treatment, which I refer to as the contemporary treatment. This made a total of 720 pots: 180 historical pots x 2 microbe treatments (live vs. sterile) x 2 contemporary watering treatments (wet vs. dry). The inoculum composed 8% by volume of the total soil with the remainder being a sterilized root-wash mix. I seeded all pots with *Brassica rapa* seeds (standard stock lines, Wisconsin Fast Plants Program, University of Wisconsin) and thinned to 1 seedling after germination. Pots were placed in a framed tray and blocked such that 2 replicates of each treatment combination existed in each frame; each treatment's location was randomized within each block. Identical to the previous watering treatment; pots were watered to saturation daily for the first week after planting to facilitate successful germination, after which moisture treatments were imposed, as described above.

In this fourth generation, I assessed the degree to which the historic selective watering and plant regimes on the soil microbial communities impacted plant adaptation to their contemporary watering environment, using multiple plant traits as our proxies for fitness, including biomass and height. Seven weeks after the initiation of this final generation, I harvested both above- and belowground biomass of the *Brassica* plants over 2 days. After harvest, I measured the height of each plant to the nearest quarter centimeter, and gently washed root tissues to remove soil particles. All tissues were oven dried at 75°C for 72 h and then weighed.

Assessing traits of soil inoculum

Characterizing soil nitrogen

While I was primarily interested in the impact of the historic selective treatments on the microbial inoculum, these treatments may have also altered the nutrition/chemistry of these soils. I tested the impact of these treatments specifically on soil nitrogen, as nitrogen seems a likely candidate for a nutrient that could be altered by these treatments while also impacting plant fitness. To this end, I conducted a KCl-extraction on a random subset of 15 soil inoculum samples within each treatment that had been collected from the end of the selection phase and freeze-dried (see above), followed by a colorimetric analysis for NH_4^+ using a modified Berthelot-salicylate method (Weatherburn 1967).

Profiling microbial community respiration over a moisture gradient

Either through ecological filtering or the evolution of the microbial community, I hypothesized that the historic watering treatment may have altered the optimal moisture range in which these microbial communities were active, becoming more adapted to their historic watering conditions. Microbial activity may be important in influencing plant tolerance to specific moisture environments (Bolin et al. 2023). Consequently, I used microbial respiration as a proxy for generalized microbial activity, and I measured the microbial respiration of a random subset of inocula (see below) across a range of soil moisture conditions. This measure is somewhat akin to the microbial communities' "moisture niche", as previously described by others (Lennon et al. 2012).

I used the MicroResp system (Campbell et al. 2003), to measure the CO_2 respiration of these microbial communities collected from the end of the selection phase. This system is

relatively high-throughput, allowing us to measure microbial respiration across a variety of samples and soil moisture contents. Briefly, I created soil microcosms by filling each well in a deep-well plate with 0.75 g of sand amended with 1.5% R2B medium (Research Products International, Mt. Prospect, IL) by weight to provide resources for microbial growth. I autoclaved and oven-dried these deep-well plates, and then added sterile water to wells across a 12-step soil water gradient, ranging from 23% to 10% gravimetric soil water content. After adding sterile water, I allowed these soil microcosms to equilibrate for 24 hours after which I inoculated each well with 15 uL of inoculum from a soil sample that had been stored at -80°C in PBS and glycerol (see previous description). I included control wells that were not inoculated with any microbial inoculum, but rather only with sterile water. I additionally prepared 96-well plates filled with a cresol red CO_2 trap gel. These CO_2 traps have a colorimetric dye that changes color according to the concentration of CO_2. Following their inoculation, these deep-well plates were clamped to the plates with the CO_2 traps using a rubber gasket, sealing each soil microcosm with a single aligned CO_2 trap well. Consequently, increased soil respiration in each soil microcosm resulted in an increased colorimetric change in the corresponding well. I read these plates' absorbance at 570 nm on a plate reader, for an initial reading. I then incubated these deep-well plates in the dark for 6 h at 25°C, following which I measured the colorimetric change of the 96 well CO_2 traps by again measuring their absorbance.

From the original 180 pots from the initial selection phase, I characterized the respiration of 60 samples, randomly choosing 10 inocula from each of the 6 historical watering and plant treatment combinations. Samples were randomly designated a location on a 96-well plate, and run over the course of 2 trials, with 4 plate systems in each trial.

Data analysis

Greenhouse biomass analysis

I used plant above- and belowground biomass, and height as our measures of plant adaptation to the contemporary watering treatments. While these measures assume that increasing biomass or height for a given watering condition represented increased plant adaptation to that environment, prior work has shown a strong correlation between *Brassica rapa* reproductive fitness and aboveground biomass (see supplemental material in Lau & Lennon, 2012). While belowground biomass or height are not frequently used as proxies for plant fitness, it can be beneficial to use multiple traits related to plant health to fully encapsulate plant fitness (Mason et al. 2017, Younginger et al. 2017). These measures may be particularly useful in evaluating plant health in relation to droughted conditions, wherein plants may differentially allocate their resource allocations to above and belowground tissues.

To examine the impact of our various treatments on plant adaptation, I constructed mixed effects models for each of these plant traits. In these models I included as fixed effects: the contemporary watering treatment (wet or dry), the microbial inoculum's historic watering treatment (wet or dry), the microbial inoculum's historic plant treatment (*Arabidopsis thaliana* (="other" plant), *Brassica rapa* (="same" plant), or no plant), as well as the status of the microbial inoculum (live or sterile). To control for variation, I additionally included the greenhouse block, and the original source pot as random effects. I squareroot-transformed these measures to meet assumptions of normality. I constructed models and evaluated the fixed effects by using the lme4 and lmerTest packages (Bates et al. 2015, Kuznetsova et al. 2015) in the R statistical environment. I used Tukey HSD *post hoc* comparisons to evaluate differences between groups.

In order to assess the impact of microbes on plant adaptation I first focused on the terms in these models representing the interaction between the microbe's historical selective treatments and the status of the microbial inoculum term (live vs. sterile). These interaction terms would indicate if live microbial inoculum was needed for any apparent plant adaptation to the selective treatments. As parsing the results of multiple plant traits can be difficult, I additionally used a separate MANOVA that combined the 3 plant traits (above- and belowground biomass, and plant height) to evaluate these treatments' impact on this suite of plant traits.

I further investigated the sign and direction of the microbial fitness impact on plants with a derived variable that quantified the difference between inoculated and sterile treatments. Every original pot from the selection phase had generated a live and sterile pair in the final testing phase. I calculated a "microbial effect" for each plant trait by dividing each live inoculum plant trait value by that of the corresponding paired sterile plant trait value (Brinkman et al. 2010). If the microbial effect is greater than 1, then the live microbial inoculum increased the plant trait, and if the microbial effect is less than 1, then the live microbial inoculum decreased the plant trait.

I used these microbial effects to create mixed-effect models similar to those described above. I included as fixed effects: the contemporary watering environment (wet or dry), historical watering treatment (wet or dry), the historical plant treatment (*Brassica* or *Arabidopsis* or no plant), and the interaction of these terms. To control for variation, I included block and the source pot as a random effects. I again additionally built a MANOVA that combined the 3 microbial effects on the plant traits. Data were log-transformed to generate log-response ratios. In all these models, I was specifically interested in the interactions involving the historical water and historical plant treatments, as our primary question was focused on the impact of plant

selection on microbially mediated plant adaptation. For example, in the contemporary dry treatment, based on prior work I expected that microbes from historical dry treatments and with plants would facilitate higher plant fitness than the historical wet treatments (Lau and Lennon 2012). However, will this benefit depend on the historic presence of the plant?

Microbial community respiration analysis

Following the methods outlined by the MicroResp manual, I transformed each well reading into a respiration rate. Using this respiration data, I characterized the range of respiration for soils from the two watering treatments across the water content gradient. Specifically, using a maximum likelihood method with the bbmle package (Bolker 2016), I generated models for respiration by fitting data to a nonlinear function that was used by others (Lennon et al. 2012) to similarly describe microbes' functional moisture profile:

$$R = R_{\max}\left(\exp\left[-\left|\frac{W - W_{\text{opt}}}{\sigma}\right|^{\tau}\right]\right)$$

The response variable in this model, R, is respiration, and is modeled as a function of: R_{max}, maximum respiration; W, soil water content; W_{opt}, soil water content corresponding to the maximum respiration (i.e., the optimum); σ, the rate at which respiration declined after maximum respiration; and τ, a shape parameter. I compared models between the two watering treatments by examining the estimates for these parameters. I specifically focused on the water content for maximum respiration (W_{opt}, optimal water content), as changes in this parameter might indicate a shift in the adaptation of these soil communities to specific water contents. I compared the breadth (defined as b) of the estimated respiration curves between historic watering treatments, calculated using various parameters from the model:

$$b = \sigma(-\log_{10} x)^{\frac{1}{\tau}}$$

where *x* defines the range of water content that is some proportion of R_{max} (Lennon et al. 2012). By assigning *x* to 0.5, this breadth parameter can be qualitatively described as the range of water content where respiration is at least 50% of the maximum respiration.

RESULTS

Plant traits

Under both contemporary wet and dry conditions, both the MANOVA and each individual plant trait (above- and belowground biomass, and height) were significantly and similarly impacted by the main effects of the soil microbial status (live vs. sterile) and the historical watering condition, but not the historical plant treatment (Table 3.1). Overall, plants were larger (both higher biomass and were taller) when paired with live microbes (Figure 3.1; Aboveground $p < 0.001$, Belowground $p < 0.001$, Height $p < 0.001$, MANOVA $p < 0.001$). Plants also tended to be larger when grown under soils that had experienced historically dry conditions (Figure 3.1; Aboveground $p < 0.001$, Belowground $p < 0.001$, Height $p < 0.001$, MANOVA $p < 0.001$), a fact that may be partially due to a more substantial draw-down of nutrients under historic wet conditions during the selection phase of the experiment (Figure B.1).

While there were several significant interactions in these models, multiple interactions across the various treatments made their effects difficult to parse. Therefore, I constructed additional mixed-effects models that were the same structure as the previous models, but split the data into the contemporary wet and contemporary dry treatments (Table B.1). As I am primarily interested in adaptation, examining models specific to each of these contemporary watering environments will allow us to evaluate specifically adaptation to either the wet or the dry environment. Within these smaller models, I found the same two statistically significant two-way

interactions across all plant traits and both contemporary watering treatments: historical watering by historical plant, and historical watering by microbial condition (See Table B.1 for details). These interactions arose because plant trait differences under historically wet and historically dry soils depended both on the historical plant and on the soil microbial status (Figure 3.1). For example, for aboveground biomass, the magnitude and direction of the historically wet vs dry treatments' impact on biomass changed across the microbial treatments. Plants grown in contemporary wet conditions largely had higher aboveground biomass when paired with live microbes from historically dry soils. Plants grown in contemporary dry conditions had significantly higher aboveground biomass when paired with live microbes from historically dry soils, but there was no difference in plant biomass under different historical watering conditions with sterilized microbial inoculum. Similar patterns were observed in the other plant trait measures (Figure 3.1). These results suggest that microbes play a positive role in plant response to dry conditions.

The microbially-mediated effect was similar across all plant traits and the MANOVA, with these traits being significantly influenced by the interaction between the historic watering treatment contemporary watering treatment, and no other factors (Table 3.2; Aboveground $p < 0.001$, Belowground $p < 0.001$, Height $p < 0.001$, MANOVA $p < 0.001$). As above, these interactions can be difficult to parse, and I, therefore, constructed two additional mixed-effects models that were the same structure as the previous models but split the data into the contemporary wet and contemporary dry treatments. In these additional models, across all plant traits and the MANOVA, in both contemporary watering environments, the historic watering treatment was highly significant, while all other factors had no significant impact on the microbial impact (See Table B.2 for all *p* values). In general, I found that soil microbes largely

were neutral or had a positive benefit to the plants (Figure 3.2; most values are at 1 or higher). Plants received the largest benefit to biomass and height from microbes when paired with microbes whose historical watering environment matched the plant's contemporary watering environment. For example, under contemporary wet conditions plants received more beneficial microbial effects with the wet microbes than the dry microbes. (Figure 3.2A-3.2C; Aboveground $p < 0.001$, Belowground $p < 0.001$, Height $p < 0.001$, MANOVA $p < 0.001$). Under contemporary dry conditions, plants received more beneficial microbial effects with the dry microbes than the wet microbes (Figure 3.2D-3.2E; Aboveground $p < 0.001$, Belowground $p < 0.001$, Height $p < 0.001$, MANOVA $p < 0.001$). Importantly, I highlight that as there was no significant interaction in these models between the historic plant and historic watering environment, the impact of the historic selective watering treatment in mediating locally adaptive plant phenotypes was not dependent on interactions with the plant.

Soil data: Microbial Moisture Niche and Nitrogen

Historic watering treatments produced different moisture niches for soil microbial communities (Table B.3, Figure 3.3). While our models predicted similar respiration for the two historic watering treatments towards the wet end of the water gradient, they significantly diverged towards the dry end of the gradient (Figure 3.3A). Specifically, models predicted significantly higher respiration at lower water contents for soils from the historic dry treatments compared to soils from the historic wet treatments. The maximum respiration for soils from the historic dry treatments occurred at a significantly lower water content than soils from the historically wet treatments (Figure 3.3B), and the niche breadth of soils from the historic dry treatments was significantly wider than that of the soils from the historic wet treatments (Figure 3.3C).

I additionally found significant differences in the nitrogen content between treatments from soils sampled at the end of the 3-generation conditioning phase (Figure B.1). Namely, there was higher nitrogen in soils from the historically dry treatments than the historically wet treatments. To determine if these differences in nitrogen content were driving plant health, I correlated nitrogen content with the residuals from the models described above (Table 3.1) but found no significant effect (Figure B.2).

DISCUSSION

I generally found that soil microbes maximally increased plant growth (i.e. microbial effect) when plants were paired with soil microbes whose historical moisture environment matched the plant's contemporary moisture environment. For example, under contemporary wet conditions, plants received maximal benefit from microbes when paired with microbes from the historical wet treatments, while under contemporary dry conditions, plants received maximal benefit to biomass from microbes when paired with microbes from the historical dry treatments. Consistent with prior work (Redman et al. 2002, Rodriguez et al. 2008, Doubková et al. 2012, Lau and Lennon 2012, Millar and Bennett 2016, Berendsen et al. 2018), these results suggest the historical moisture environments selected for microbes that mediate plant local adaptation to the selective moisture environment. Importantly, I observed this same pattern of microbial fitness benefits across all historical selective plant treatments, including those that did not include a historical association with a plant. This suggests that wet/dry microbial fitness benefits to plants emerge as an incidental byproduct of selection on these microbes from the moisture environment and not as part of a co-adapting plant-microbe system.

Why should selection for (e.g.) dry-adapted microbes result in fitness benefits for plants under dry conditions? One possible explanation is that the moisture environment may select for microbes with traits that both benefit themselves and also incidentally benefit plants as a byproduct. For example, dry soil conditions may select for increased microbial biofilm production, because microbial biofilms can prevent microbial desiccation (Lennon et al. 2012). While these biofilms are a direct microbial response to drought, microbial biofilms can also support plant drought tolerance if the biofilms develop as a rhizosheath around plant roots, protecting the roots from desiccation (Naseem et al. 2018). Thus, while the production of these biofilms may be a selfish trait from the perspective of the microbes, they incidentally positively impact the fitness of the plant (i.e., byproducts/incidental cooperation, see Sachs *et al.*, 2004).

Another possible explanation is that soil microbes locally adapted to the contemporary watering environment may maximize plant fitness by preventing the dormancy of beneficial microbes. This maintains crucial microbial functions that are necessary for plant health. Indeed, in our study (as well as many others) plants were healthier when inoculated with live soil compared to sterile soil, highlighting the general benefit to the plant of the soil microbial community. These broadly beneficial microbes may be involved in nutrient cycling, decomposition, pathogen suppression, etc. (Lugtenberg and Kamilova 2009, Ahemad and Kibret 2014); some of these broadly beneficial traits likely evolved for the microbial benefit, and not the plants', with any benefit incidental to the plant. Each of these beneficial microbial taxa likely has a range of environments within which they are physiologically active (Rath et al. 2019); if the current environment is outside a microbe's range, that taxa likely becomes dormant in the community or becomes locally extinct (Lennon and Jones 2011). The historic watering treatments may select for these beneficial microbes locally adapted to those conditions, causing

these microbes to be available to aid the plant; for example, under contemporary dry conditions, microbes with dry-adapted traits may be available to benefit and influence the plant, while wet-adapted microbes are dormant.

This second explanation receives some support from our observed microbial respiration data, because the optimal respiration for the microbial communities from the historical dry treatment was at a lower water content than the wet treatments. Moreover, these dry communities had higher respiration relative to the wet communities under lower soil moisture conditions, and they had a wider respiration breadth. To the extent that soil respiration is an indicator of overall microbial activity, these differences in respiration may indicate that the historic dry treatments led to the adaptation of these microbial communities to lower soil water contents; thus, these dry-adapted microbes may have been better able to continue to provide beneficial functions to plants under low water conditions. I do note however, that these respiration measures may also be limited because a) these differences in respiration were quite small relative to the overall variance of respiration rate and b) total respiration broadly profiles microbial activity across this water gradient, and I do not know which particular microbial taxa were the main responders in respiration, nor do I know how they influence plant health. Isolating specific microbes from these soils and assessing their functional capacity under a range of moisture conditions could follow up and test these hypotheses.

While thus far I have used the term “selection” to refer to potential changes in soil microbes by the environment, is unclear if these results are the product of natural selection (e.g. microbial evolution), ecological selection (i.e. filtering), or even shifts in microbial dormancy of the community. In the context of soil microbial communities, with short generation times, rapid mutation rates, and frequently dormant microbes, distinguishing between these is not possible

without molecular tools. Indeed, I unfortunately conducted no sequencing in this study. Sequencing could potentially identify microbial mechanisms influencing the plant adaptation observed herein. Specifically, I suggest that future work addressing similar questions could use shotgun metagenomic-based approaches to identify specific microbial genes under selection or could use culture-based approaches to track the evolution of model microbes.

This experiment was designed to investigate the impact of selection on the microbes, and that selection's subsequent impact on the plant. Consequently, I generated a microbial effects index that compared fitness measures between the live and sterile treatments. However, a caveat to our study is that even within the sterile treatments, there were significant differences between the historical watering treatments. For example, this was particularly notable in the contemporary wet greenhouse treatments, inoculated with sterile soils selected upon by *Brassica* under the historically dry conditions, which had substantially higher plant biomass than any other sterile inoculum. Differences in plant performance within the sterile soil treatments were potentially related to the changes in soil nutrition/chemistry during the selection phase. For example, I note that soils from historically wet conditions had lower nitrogen than soils from historically dry conditions in the two plant-containing treatments (Figure B.1). This is likely to reflect greater nitrogen uptake by well-watered plants during the selection stage, and this might lead to soil fertility differences that influenced plant growth during the final stage of our experiment. I did not find that soil nitrogen correlated with plant biomass (Figure B.2), but it is hard to rule out all possible soil chemical/nutritional changes due to these historic treatments without an extensive profile of soil properties. I note that the high fitness of the droughted *Brassica* soil compared to the no plant treatments suggests that *Brassica* is somehow promoting future plant growth through an abiotic means, though the mechanism is unclear. Because our microbial effects index

was generated from paired samples, I argue that pre-existing nutritional/chemical changes from historical treatments should be carried over to both live and sterile inocula. Thus, nutritional/chemical changes to the soil should be controlled for in the microbial effects index, and any remaining differences between treatments can be attributed to changes in the soil microbes. I found a clearer signal of historical-contemporary environmental matching in the microbial effects analysis (Figure 3.2), suggesting that microbes are largely responsible for wet-dry fitness benefits.

CONCLUSION

Our work suggests that the soil moisture environment, independent of the plant, selects for soil microbial communities that maximize plant biomass when under the same moisture environment. These results, as well as that of others, emphasize the significant impact of microbes on their host's fitness and suggest that these plant-microbe interactions may frequently play an adaptive role for the host, and may be influential in shaping each partner's evolution (TerHorst et al. 2014, Osborne et al. 2018, Petipas et al. 2021). Importantly, this work may indicate that, at least in the timeframe of this study, the plant may be relatively unimportant in guiding the functional evolution of some of its microbial partners, with any beneficial effects of the microbes to the plant only an incidental byproduct of the selective abiotic environment. I do emphasize however that many microbes coevolve with their partners, including rhizobia and mycorrhizal fungi (Hoeksema 2010, Batstone et al. 2020a), neither of which is associated with the plant species I used. Such microbes however are a part of some of the most intricate plant-microbe symbioses, and they may not be representative of the plant interactions with the diffuse soil microbial community.

As many microbial processes benefit their host plants, it may be intuitive to use an adaptationist paradigm to explain the emergence of these beneficial microbes, with these beneficial microbes being selected with the express purpose of aiding the plant. However, our results highlight that some of these locally adaptive and beneficial microbes may arise independently of the plant, with any benefit to the plant only as a byproduct of environmental selection.

Host-microbe interactions appear ubiquitous, with all eukaryotic organisms apparently colonized by large communities of microorganisms (Gordon et al. 2013, Simon et al. 2019). Given their frequent significant impact on host adaptation, I need a strong understanding of the selective agents on these host-associated microbes, and their impact on host adaptation. Future studies can follow our work herein by explicitly examining the microbial traits that underpin host fitness and phenotype, and investigating the selective drivers of these traits.

FIGURES AND TABLES

Figure 3.1: Various plant traits from *Brassica rapa* plants in our greenhouse experiment, including aboveground biomass ((a) and (d)), belowground biomass ((b) and (e)), and height ((c) and (f)). Data are grouped by the present greenhouse watering treatment (wet, panels (a), (b), and (c), or dry, panels (d), (e), and (f)), the microbial inoculums historic watering treatment (wet or dry, coded as blue or orange), the microbial inoculum's historic plant treatment (*Arabidopsis thaliana* or *Brassica rapa* or no plant), as well as the status of the microbial inoculum (live or sterile). Data were square-root transformed in our analysis, but were back-transformed here for interpretation. We present here estimated marginal means, with bars representing 95% confidence intervals generated from the standard error. Lettering represents *post hoc* Tukey HSD, with groups that differ in their lettering being significantly different from one another. As we are primarily interested in adaptation to each contemporary water environment, comparisons across the contemporary watering treatments may not be informative. We therefore based *post hoc* comparisons off the reduced models (see Table B.1) representing each contemporary watering environment.

Figure 3.1: Continued

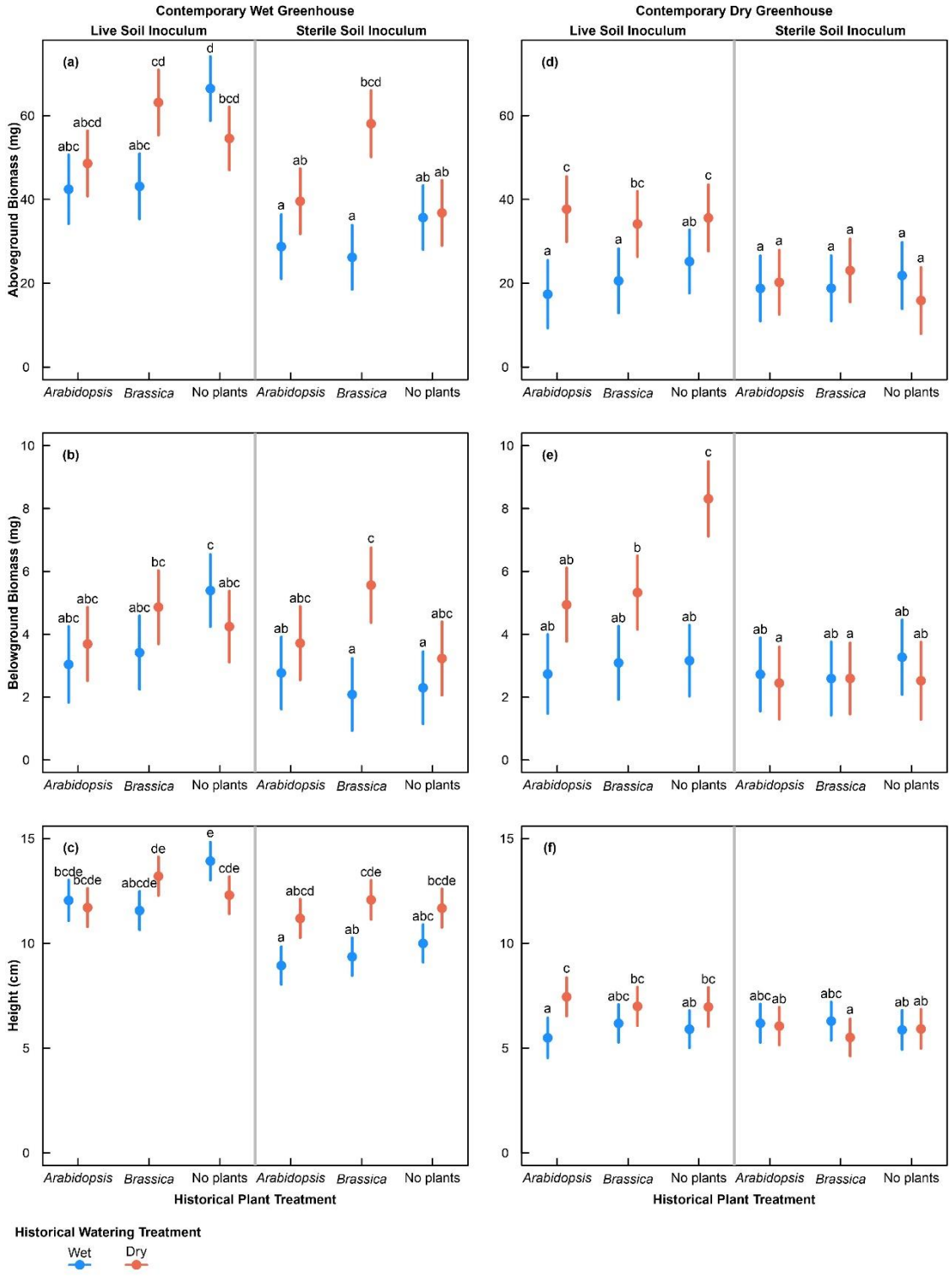


Figure 3.2: Impact of microbes on *Brassica rapa* various traits in our greenhouse experiment, including aboveground biomass ((a) and (d)), belowground biomass ((b) and (e)), and height ((c) and (f)). Microbial impact is calculated as the log-response ratio of the plant trait from plants provided with live inoculum vs. the plant biomass from plants provided with sterile inoculum. The main effects of the historic watering environment on each trait are presented, though we display their interactions with the historic plant treatments with transparent lines. Data are grouped by the present greenhouse watering treatment (wet or dry, panels (a), (b), and (c), or dry, panels (d), (e), and (f)), the microbial inoculums historic watering treatment (wet or dry, coded as blue or orange), and the microbial inoculum's historic plant treatment (*Arabidopsis thaliana* or *Brassica rapa* or no plant). The dashed grey line at 1 is provided as reference for the impact of the microbes. We present here estimated marginal means, with bars representing 95% confidence intervals generated from the standard error. The statistical significance of each comparison is indicated using the symbology as follows: ns $p > 0.10$; + $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

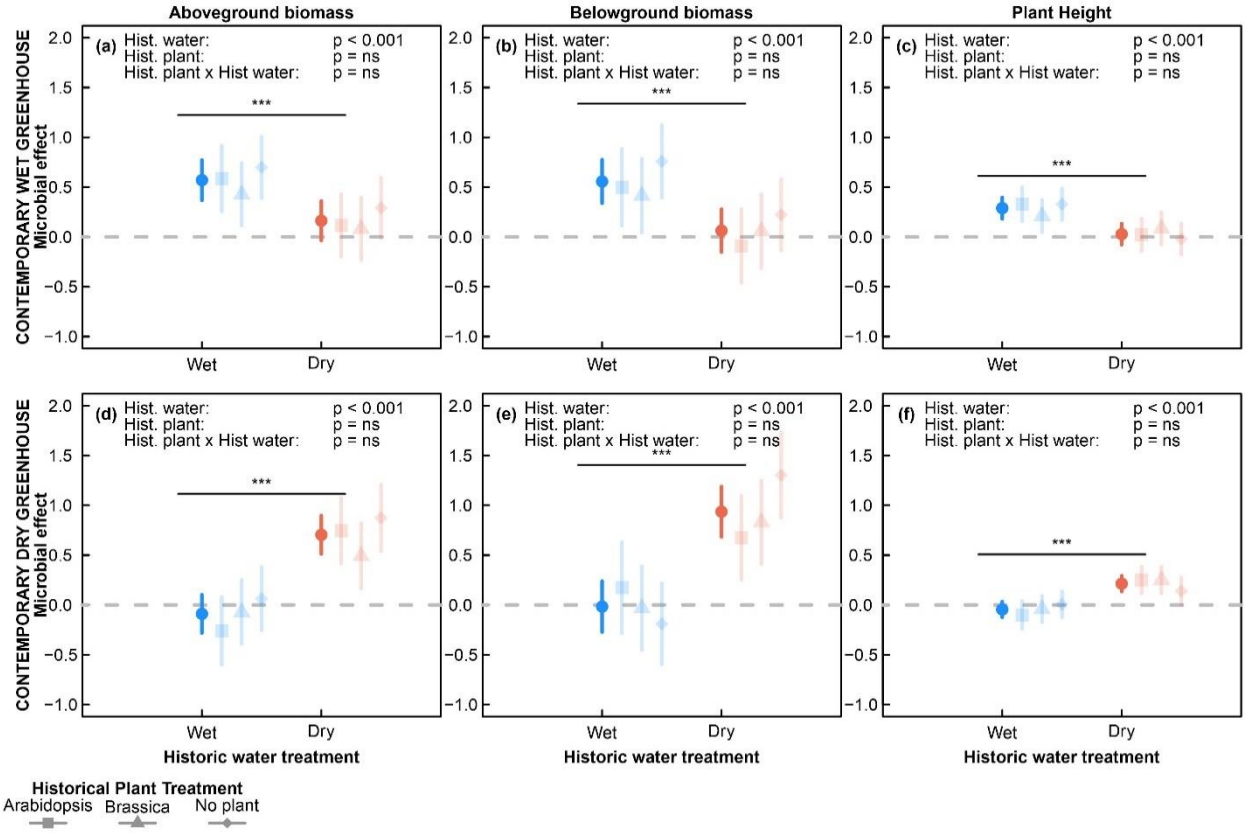


Figure 3.3: Models for microbial respiration across a soil moisture gradient. Separate models were constructed for the two historical soil treatments, with the soil microbes from the historic dry treatments denoted in orange and the soil microbes from the historic wet treatments denoted in blue. a) The curves for the maximum likelihood models fit to the data using a biologically relevant model described in Lennon *et al.* (2012). Confidence intervals over the prediction interval were estimated via bootstrapping. Mean respirations of each treatment group at each water content level are displayed with solid points, while the actual respiration data are displayed with semi-transparent points. Data were jittered along the x-axis for ease of viewing. b) Estimates for the niche water optimum (based on the gravimetric soil water content) for the two historic watering treatments. These values were parameters estimated as a part of the models. c) Estimates for the niche breadth for the two historic watering treatments. These values were estimated based on various parameters extracted from the models, as described by Lennon *et al.* (2012), and can be described as the range of water content where respiration rate is 50% of the maximum respiration.

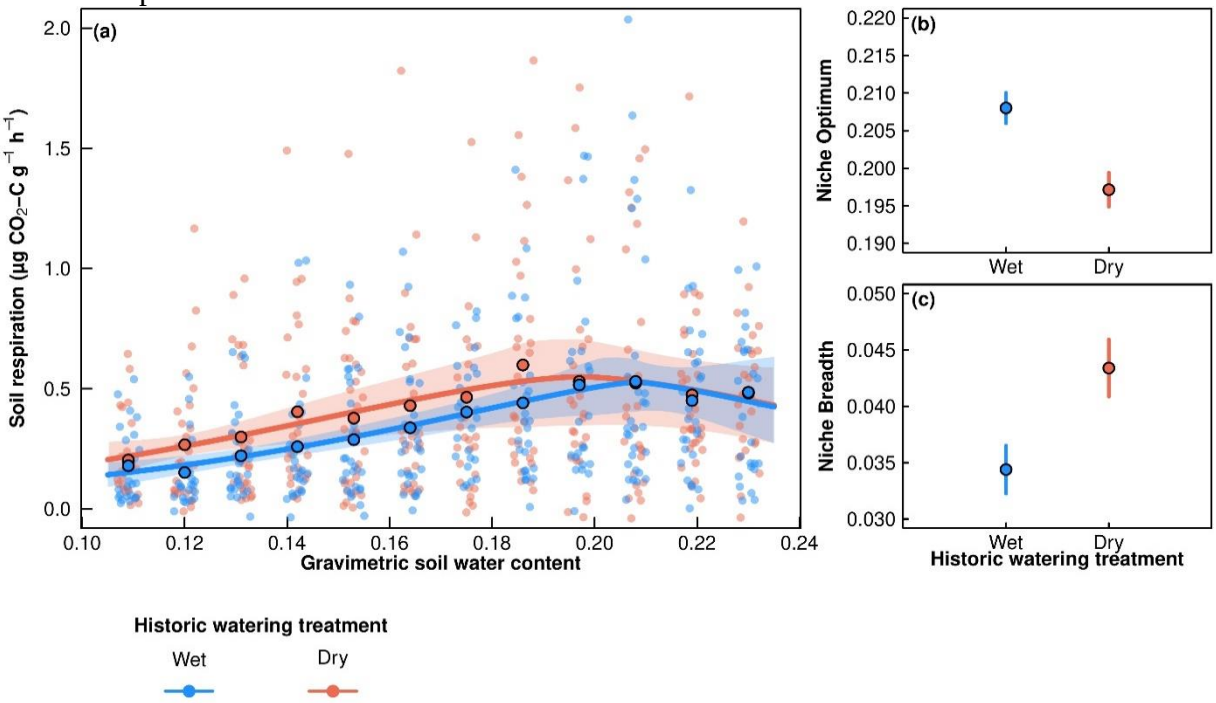


Table 3.1: Mixed model ANOVAs for the effects of the contemporary watering treatment (wet vs. dry), historic plant conditioning treatment (*Arabidopsis thaliana* vs. *Brassica rapa* vs. no plant), historic watering treatment (wet vs. dry), the microbial status (live vs. sterile), and their interactions on multiple plant traits, including above- and belowground biomass, as well as height. I additionally show a MANOVA that combines all 3 plant traits together. I display the F value for each model term. Terms with an associated *p* value less than 0.05 are bolded. + $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Model Term	df	Aboveground Biomass	Belowground Biomass	Plant Height	MANOVA
Contemporary Water	1	**107.11*****	0.34	**550.92*****	**292.19*****
Historic Water	1	**36.16*****	**32.43*****	**16.77*****	**13.04*****
Historic Plant	2	2.78^{+}	**3.59***	1.26	1.98^{+}
Microbe	1	**67.72*****	**40.78*****	**39.01*****	**24.16*****
ContWater x HistWater	1	0.19	0.12	1.44	1.50
ContWater x HistPlant	2	1.28	0.71	2.34^{+}	**2.87****
HistWater x HistPlant	2	**10.04*****	1.66	1.32	**5.27*****
ContWater x Microbe	1	1.93	2.97^{+}	**8.62****	**9.39*****
HistWater x Microbe	1	2.69	**4.97***	0.23	2.43
HistPlant x Microbe	2	**3.49***	**3.37***	0.11	1.91^{+}
ContWater x HistWater x HistPlant	2	**3.53***	**3.32***	**4.90****	**3.04****
ContWater x HistWater x Microbe	1	**26.03*****	**29.82*****	**32.36*****	**14.65*****
ContWater x HistPlant x Microbe	2	0.12	0.09	0.24	0.53
HistWater x HistPlant x Microbe	2	0.51	0.72	1.47	1.40
ContWater x HistPlant x HistWater x Microbe	2	0.41	2.08	0.73	1.25

Table 3.2: Mixed model ANOVAs for the effects of the contemporary water treatment (wet vs. dry), historic plant conditioning treatment (*Arabidopsis thaliana* vs. *Brassica rapa* vs. no plant), the historic watering treatment (wet vs. dry), their interactions on the microbial effect on multiple plant traits, including above- and belowground biomass, as well as height. I additionally show a MANOVA that combines all 3 plant traits together. Note, that these models contrast from the previous models as there is no 'Microbe' term as the response variable here was the effects of the microbes on biomass (the ratio of plant biomass provided with live soils vs. plant biomass provided with sterile soils). This table includes the results from the separate models for plants under the contemporary wet treatment and those under the contemporary dry treatment. I display the F value for each model term. Terms with an associated *p* value less than 0.05 are bolded. + $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Model Term	df	Aboveground Biomass	Belowground Biomass	Plant Height	MANOVA
Contemporary Water	1	0.22	3.06^{+}	2.83	2.40^{+}
Historic Water	1	0.86	1.35	0.01	0.11
Historic Plant	2	2.65	1.13	0.05	0.82
ContWater x HistWater	1	**37.94*****	**34.28*****	**39.28*****	**15.08*****
ContWater x HistPlant	2	0.16	0.12	0.34	0.54
HistWater x HistPlant	2	0.01	1.56	1.77	1.14
ContWater x HistWater x HistPlant	2	1.22	1.93	0.76	0.94

CHAPTER 4: SYMBIOSES AS ENGINES FOR ADAPTATION? RHIZOBIA EVOLUTION FACILIATES RAPID PLANT ADAPTAITON TO WATER STRESS

ABSTRACT

While a host's associated microbial community can frequently influence host phenotype, these microbes can have temporally variable impacts on the host due to their rapid evolution. To link host phenotype and their microbial symbionts' evolution, I investigated selection from the interactive host and abiotic environments on host-associated microbial evolution. I specifically used the legume-rhizobia mutualism as a model symbiosis, conducting a long-term evolution experiment wherein I evolved populations of rhizobia under variable watering conditions. I crossed these treatments with two plant treatments, *Trifolium repens* present and absent, thus varying host selection on the microbe. After four plant generations, I evaluated the role of these selective treatments in mediating microbes with adaptive benefits to the plants by re-isolating rhizobia strains and inoculating the evolved rhizobia strains back onto *Trifolium* under the original watering conditions. I generally found that rhizobia maximally increased plant growth when plants were paired with rhizobia that had evolved with a plant and under moisture conditions that matched that plant's contemporary moisture environment. Importantly there was little variation in plant growth measures when inoculated with the rhizobia that did not evolve with a plant. These results suggest that selection from the plant drove the evolution of rhizobia beneficial to the plant in their current moisture environment. Indeed, the rhizobia symbiosis did not collapse in the face of stress, but rather, the symbiosis rapidly adapted to its current environment, serving as an engine for host local adaptation.

INTRODUCTION

While a significant proportion of an organism's phenotype can be attributed to heritable genetic variation, many of the drivers of phenotype remain unexplained (Kaprio 2012, Lind et al. 2018, Young 2019). Given that plants and animals are host to complex communities of microbial symbionts who, through their various activities and traits, can alter their host's phenotype and consequently their degree of adaptation to the environment (Friesen et al. 2011, Petipas et al. 2021, Ricks et al. 2023), recent work has suggested that some variation in phenotype and adaptation may be explained by an organism's interactions with their associated microbial symbionts (Sandoval-Motta et al. 2017). However, due to their short generation times, these symbiotic microbes' interactions with their hosts can rapidly evolve, shifting between beneficial and parasitic interactions over ecologically relevant time scales (Drew et al. 2021, Li et al. 2021). As organisms across all biological levels of organization can be impacted by these microbial symbionts, understanding the drivers of these symbiotic microbes' evolution, specifically concerning their impact on their host's adaptation, may shed light on the drivers of host phenotype across a variety of systems.

As adaptation is an inherently local concept (Kawecki and Ebert 2004, Blanquart et al. 2013), any microbial impact on host phenotype and adaptation, whether beneficial or detrimental, may be dependent on the given abiotic environment. For example, many microbial symbionts can influence their host through the provisioning of resources (Schwartz and Hoeksema 1998, Sachs et al. 2004, Wyatt et al. 2014); these interactions can therefore be context dependent as shifts in the abiotic environment can alter the availability of resource exchanged, with resources beneficial in one environment potentially providing little value in another. Moreover, the selective abiotic environment may interact with the microbe's host as the host can

influence microbial fitness through access to resources and habitats. Therefore, shifts in the abiotic environment may alter a microbe's fitness as a consequence of its interaction with the host, and thus may impact the evolution of microbial traits that influence host adaptation in a given environment.

A given abiotic environment may select for increasingly locally beneficial and mutualistic microbial interactions. As host symbionts can be dependent on their partners for essential resources, there may be selective pressure for microbial traits that facilitate host stress tolerance and adaptative phenotypes. Indeed, while many microbial traits may be viewed as costly to the microbe, these traits can be selected for and maintained in the microbial population as they may increase host resources allocated towards the microbe and thus increasing their fitness (Westhoek et al. 2017, Oono et al. 2020). Coevolutionary mechanisms between the microbial symbiont and the host may therefore interact with the host's abiotic environment to drive the rapid evolution of microbial traits that mediate locally adaptive host phenotypes.

However, the abiotic environment may alternatively select for microbial partners with maladaptive impacts on their host. As these microbial interactions can be context dependent across various environments, some stressful environments may move otherwise beneficial and mutualistic symbioses into unstable zones, thus selecting for microbes with potentially maladaptive effects on their hosts. For example, warming oceans, a consequence of climate change, has led to the large-scale stand failure in coral as a result of ineffective coral mutualistic symbionts under increased thermal stress (Warner et al. 1999, Hoogenboom et al. 2012). Therein, thermal stress altered the effectiveness of the symbionts, driving the coral hosts to eject their partners. Stressful abiotic environments may thus select for maladaptive microbial symbionts.

To evaluate the selective pressures on a microbial symbiont's evolution, I used leguminous plants and their interactions with rhizobia as a model system. Rhizobia are a group of bacteria that colonize nodules on the roots of leguminous plants, wherein they fix atmospheric nitrogen into a biologically available form which they exchange for photosynthetically derived carbon from their host plant. This symbiosis may be a model system to address these questions given there has been extensive prior work identifying the rhizobia traits driving nitrogen exchange with their host (Shamseldin 2013, Epstein et al. 2018), as well as the feasibility of isolating isogenic strains of rhizobia and inoculating them onto greenhouse-grown plants. I specifically used a long-term greenhouse experiment to experimentally evolve rhizobia under various host and abiotic environments for multiple plant generations. Namely, I evolved these rhizobia under two moisture treatments, wet vs. dry, representing an unstressed vs stressed environment respectively. I initially hypothesized that these droughted environments may either select for rhizobia partners that mitigate drought stress, or alternatively, the stress would destabilize the mutualism and select for increasingly maladaptive rhizobia partners. As these hypotheses may be predicated on coevolutionary interactions with the host, with potential interactions between the abiotic environment and plant investment into the rhizobia symbionts, I included two plant treatments, a plant present and a plant absent treatment. These two plant treatments differed in the presence of the host plant, allowing us to evaluate the contribution of host selection and its interaction with the abiotic environment on microbial evolution. I additionally included two nitrogen treatments, a nitrogen addition and a no nitrogen control. This nitrogen treatment acted as a control, as a known stress on the legume-rhizobia system, as prior work has shown that nitrogen degrades the quality of the rhizobia symbiosis (Weese et al. 2015). Following these selective treatments, I isolated rhizobia from these populations, and inoculated

them back onto plants under the two original watering treatments, allowing us to evaluate the relative importance of the host and the abiotic stress in selecting on these rhizobia partners' evolution, and their impact on plant adaptation. If plants are selecting for rhizobia partners with a locally adaptive benefit, then I would predict that rhizobia that evolve with a plant from a given moisture environment will benefit the plant the most in that moisture environment. Conversely, if increased stress destabilizes the mutualism, then I would predict that rhizobia that evolved under the droughted conditions would be less beneficial to the plant.

MATERIALS AND METHODS

Rhizobium Experimental Evolution

Selection Phase (Generations 1–4)

I established a long-term greenhouse selection experiment to investigate the selective agents on rhizobia in their impact on mediating locally adaptive or maladaptive plant phenotypes. For four plant generations, I grew rhizobia populations under multiple selective treatments, among these including included two moisture treatments, either wet or dry conditions, which I refer to as the historic moisture selection treatment. I crossed these moisture treatments with two plant treatments, with a leguminous plant present or those without a plant, which I refer to as the historic plant treatment. Altering the presence of plants in this experiment altered the presence of plant selection on the microbes. Finally, I crossed these treatments with two nitrogen treatments, nitrogen addition or no nitrogen addition, which I refer to as the historical nitrogen treatment.

In the first generation, I established soil mesocosms by filling pots to near capacity with approximately 430 g of a locally produced "root-wash" soil mix: equal parts of calcined clay, torpedo sand, and field soil (University of Illinois Plant Care Facility,

https://pcf.aces.illinois.edu/soil-mixes/). There were 15 replicate pots in each treatment, for a total of 120 pots (15 reps x 2 watering treatments x 2 plant treatments x nitrogen treatments). At the start of each of the first four generations, I planted approximately 100 seeds in pots designated in the 'plant present' treatment, using white clover seed from a seed supplier (*Trifolium repens* L. sourced from Ernst Seeds, Meadville, PA, USA). As seeds came from a supplier, mesocosms were likely filled with a variety of clover genotypes. To prevent the introduction of outside rhizobia, I sterilized the seed exteriors by washing them in 3% sodium hypochlorite and 0.01% Tween-20 solution for 2 minutes, followed by rinsing them in sterile water. At this time, I additionally amended these mesocosms with nitrogen to those designated the 'nitrogen addition' treatment. Nitrogen addition treatment amounted to 0.03 g of ammonium nitrate to each assigned pot at the start of each generation. These N-addition levels were scaled to be equivalent to the highest potential rates of annual atmospheric N-deposition, as well as the N-fertilization levels in agricultural settings (Martinelli et al. 2008). Moreover, I chose these levels in part to be identical to those applied in a long-term nitrogen deposition experiment at the Kellogg Biological Station (Huberty et al. 1998, Dickson and Gross 2013, Weese et al. 2015). I included a rhizobia population that could be the target of selection by the various treatments by inoculating each pot with a rhizobia inoculum at the start of the first generation. This inoculum was generated by pooling 28 strains of *Rhizobium leguminosarum* previously isolated from the Kellogg Biological Station (Weese *et al.*, 2015). Isolates of each strain were grown separately in 10 mL of liquid TY medium (0.5% tryptone, 0.3% yeast extract, and 10 mM $CaC1_2$) at 30°C with shaking. After 2 days of growth, I diluted strains to a standard optical density (OD_{600}) of 0.1 and combined them in equal concentrations. Upon preparation, these rhizobia were immediately inoculated onto mesocosms in the greenhouse, adding 10 mL to each pot.

At the start of each generation, all pots were watered to saturation daily for 1 week after sowing to facilitate successful germination and plant establishment, after which the moisture treatments were imposed: in the wet treatment, pots were watered to saturation every 2 days while in the dry treatments, I weighed pots and watered to 14% gravimetric water content every 2 days. I chose this water content target for the dry treatment as a pilot study demonstrated that it represented a significant stress compared to the well-watered control, while still allowing for plant survival. These moisture treatments thus allow for differing selective environments, which can be used to evaluate plant adaptation to each moisture environment. Plants were grown in the greenhouse on a 26°C/24°C day/night schedule, supplemented with 14 h of daily light. I continued these treatments for a total of 4 plant generations, with each generation lasting approximately 10 weeks. Between each generation, plant shoots were pruned, and pots were allowed to remain fallow for 3 weeks. As senescing nodules release rhizobia cells back into the soil (Denison and Kiers 2006), this step allowed for the plant-associated rhizobia to re-enter the soil population and be available for the next generation of plants.

Isolation

After 4 plant generations of selection on these rhizobia populations, I evaluated these populations' evolution by isolating rhizobia from these populations. While I would ideally isolate rhizobia directly from the soil, this can be prohibitively difficult. Consequently, most prior work has isolated rhizobia directly from plant nodules. Given that I included treatments that did not contain any plants with nodules, I included a fifth plant generation in the greenhouse, wherein I could use plants to trap rhizobia for isolation in nodules. To this end, identical to the start of the selection experiment, I prepared new pots filled with sterile soil and 100 surface

sterilized clover seeds (see above). Then, I sampled 30 g of soil from each of the original mesocosms which I diluted in 200 mL of a sterile 0.9% NaCl solution to make a soil slurry. After 1 week of growth in the greenhouse, I inoculated 5 mL of these soil slurries onto the new plant populations. These plants were all watered to saturation every 2 days, under identical climate conditions as the prior experiment, for 5 weeks in the greenhouse, after which they were harvested for nodule isolation.

From each pot, I randomly selected 6 plants for isolation. On each of these plants, I chose for isolation those nodules closest to various predetermined haphazard locations on the root system to avoid biasing the sampling by nodule size. The selected nodules were surface sterilized with bleach and ethanol, squashed to expose rhizobia, and plated onto TY agar (Heath and Tiffin 2009). Plates were incubated at 30°C, and strains were serially plated to isolate individual colonies. Each strain was then propagated in liquid TY media at 30°C for 48 h and frozen in a 75% TY and 25% glycerol solution at -80°C for future use. I verified the identity of these rhizobia using sequencing. Specifically, I PCR amplified the 16S region using 8F and 1492R primers and submitted samples for both forward and reverse Sanger sequencing at the Roy J. Carver Biotechnology Center (Urbana, IL, USA). I ultimately identified 446 *Rhizobium* from this experiment.

Assessing evolved rhizobia on plant adaptation

To assess the impact of rhizobia evolution from these selective treatments in mediating plant adaptation to variable moisture environments, I conducted a final greenhouse experiment, evaluating plant adaptation to both wet and dry conditions using evolved single rhizobia strains as the plant's partners. From the rhizobia strains I had isolated, I randomly selected 320 strains to

evaluate, with 40 strains from each of the 8 treatments, and spread relatively evenly across the original populations. Following methods similar to those described above, I filled sterilized "cone-tainer" pots with autoclaved sterilized root-wash soil mix. For each strain, I planned to grow 3 replicate plants in each watering treatment, while also including 20 plants not provided any rhizobia inoculum as a control, for a total of 1960 plants (2 watering treatments x 320 strains x 3 replicates + 40 control (20 wet and 20 dry plants)). Using the same seed as from the initial selection phase (sourced from a seed supplier), I sprinkled approximately 10 surface sterilized clover seeds in each pot, and then watered pots to saturation every other day to facilitate successful germination and plant establishment. As plants germinated, I thinned each pot to a single seedling. One week after the seeding, I inoculated plants with their specific rhizobia strain and imposed the moisture treatments. To inoculate plants, I grew the selected rhizobia strains in liquid TY media at 30°C, 48 h prior to inoculating these plants. Using these liquid cultures, I then standardized the number of rhizobia cells for each strain by diluting each culture to a standard optical density of 0.15 (OD_{600}), after which I inoculated each pot with 1 mL of the designated strain. Similar to the initial evolution experiment, I imposed moisture treatments by, every 2 days, watering plants assigned to the wet treatment to saturation while watering those assigned to the dry treatments to a 14% gravimetric water content. Unfortunately, there was a small outbreak of grasshopper herbivory in the greenhouse in the middle of this experiment. After eradicating the outbreak, I recorded which plants were grazed upon (approximately 5%) and quantified the damage, to be used as a factor in subsequent models.

Five weeks after the initiation of this experiment, I measured plant leaf number as a preliminary measure of plant health. Eight weeks after the initiation of this experiment, I harvested both the above- and belowground biomass of the *Brassica* plants over the course of 2

days. Aboveground tissues were measured for their height (to the nearest quarter centimeter), oven-dried at 75°C for 72 h, and then weighed. I gently washed belowground tissues of their soil, and immediately stored them in a -20°C freezer. As a proxy for rhizobia fitness, I dissected belowground tissues to count the number of root nodules (Batstone et al. 2017, Gano-Cohen et al. 2019, Zhang et al. 2020), after which they were similarly oven dried and weighed for their mass.

Data analysis

Similar to other studies in the legume-rhizobia system, I measured a variety of plant traits to capture the degree to which the rhizobia mediated locally adaptive plant phenotypes to their contemporary watering environment, including plant above- and belowground biomass, leaf number, and height (Forrester et al. 2020, Heath et al. 2020, Vaidya and Stinchcombe 2020). I assume increases in these measures represented increased plant fitness. While traditional metrics of plant fitness typically use seed or fruit numbers, this target organism can take months to flower and produce seed, making using these fitness proxies a necessary limitation of this study. While belowground biomass or height are not frequently used as proxies for plant fitness, it can be beneficial to use multiple traits related to plant health to fully encapsulate plant fitness (Mason et al. 2017, Younginger et al. 2017). Moreover, root biomass may be a particularly useful metric, as plants may increase allocations to belowground tissues under drought-stressed conditions (Eziz et al. 2017), as well as when their rhizobia do not provide sufficient nitrogen resources (Friesen and Friel 2019, Wendlandt et al. 2022), allowing plants to more effectively scavenge water and nutrients.

I first evaluated potential contamination and the sterility of the treatments by comparing all plant traits as well as nodule number between live and sterile controls. Plants assigned to the sterile control, inoculated with no rhizobia, were on average significantly smaller in every plant health metric (above- and belowground biomass, height, and leaf number) than those inoculated with live rhizobia, under both the contemporary wet and dry treatments (Figure C.1). However, some of these sterile control plants did have a small degree of nodulation, despite not being provided with any inoculum and growing in sterile soils (Figure C.1e, C.1j), indicating some degree of cross-contamination in the greenhouse. I do emphasize that this contamination however was minimal, with control plants from the contemporary wet treatments producing 13% as many nodules as those inoculated with live rhizobia (on average 10.8 and 80.6 nodules, respectively), and with control plants from the contemporary dry treatment producing 6% as many nodules as those inoculated with live rhizobia (on average 2.5 and 29.8 nodules, respectively). As the experiment was randomized, this contamination was likely random with respect to treatment. These patterns of minimal, but present, contamination are frequent in many prior rhizobia studies, though such contamination does not erode significant treatment variation (Weese et al. 2015, Regus et al. 2017, Heath et al. 2020). Using a similar interpretation as prior work, I suggest that such contamination likely will minimize variation between treatments, with significant results suggesting that these selective treatments may be strong drivers of rhizobia evolution and subsequent plant adaptation, even in the face of contamination. In all subsequent analyses I excluded plants that were inoculated with the sterile control treatment.

To examine the impact of the various selective treatments on these plants' adaptation, I constructed mixed effects models for each of these plant traits. These included separate models for each of the plant traits, as well as the root-shoot ratio, as a measure of plant investment into

belowground resources. In these models I included as fixed effects: the contemporary watering environment (wet or dry), the rhizobia's historical historic moisture treatment (wet or dry), the rhizobia's historic plant treatment (plant present or no plant), and the historical nitrogen treatment (no nitrogen addition or nitrogen addition). To control variation, I included the greenhouse block and herbivory level as random effects. I constructed models and evaluated the fixed effects by using the lme4 and lmerTest packages (Bates et al. 2015, Kuznetsova et al. 2015) in the R statistical environment. As parsing the results of multiple plant traits can be difficult, I additionally used a separate MANOVA that combined the 4 plant traits (above- and belowground biomass, leaf number, and plant height) to evaluate these treatments' impact on this suite of plant traits. Root-shoot ratio was excluded from this MANOVA as it is derived from other variables included in the model, while also not directly related to plant health.

Similar to other work (Batstone et al. 2017, Gano-Cohen et al. 2019, Zhang et al. 2020), I used rhizobia nodule number as a proxy for rhizobia fitness, because senescing nodules release rhizobia cells back into the soil (Denison and Kiers 2006), relating nodule number directly to rhizobia fitness. Nodule number does ignore variation in nodule size, though in a subset of 100 plants randomly chosen across treatments I found that total nodule biomass was strongly correlated with nodule number (see Figure C.2). Limited resources and time prevented me from measuring nodule biomass for all 2000 plants. I evaluated the impact of these selective histories on nodule number by building similar mixed effects models to those described above, with the exception that I included the individual who counted nodules on a given root system as a random effect.

I characterized the impact of these selective treatments on the stability of the rhizobia-legume mutualism by evaluating the correlation between rhizobia nodule number and plant

proxies for fitness. The stability of a mutualism may be maintained through commensurate benefits between partners, often observed with an alignment in fitness between partners. Misalignment in fitness suggests that some partners are "cheating", having high relative fitness while providing little benefit to their partners (Friesen 2012, Jones et al. 2015). To this end, we can determine which treatments are selecting for ineffective or cheating partners by examining the correlation between rhizobia and plant fitness estimates between the different rhizobia histories (Jones et al. 2015). As I had multiple metrics of plant health, I created a single measure by generating principal component axes of the aboveground plant traits (see Table C.1 for PC loadings). To evaluate differences in this plant-rhizobia fitness correlation between treatments, I constructed a model with the first PC axis as the response variable and nodule number as the explanatory variable, specifically including the rhizobia historical treatments as interactions. Significant terms in this model that involved the historical treatments interacting with nodule number would indicate different slopes in this plant-rhizobia fitness correlation between selective treatments, thus changes in fitness alignment. I constructed separate models for contemporary wet and dry conditions.

RESULTS

All traits (aboveground, belowground, leaf number, height, nodule number, and root-shoot ratio) as well as the MANOVA were significantly and similarly impacted by the main effects of the contemporary watering treatment (Table 4.1). Unsurprisingly, plants were larger (both higher biomass, taller, and produced more leaves), had more nodules, and had a smaller root-shoot ratio when grown under wet conditions compared to dry conditions (Table 4.1; Aboveground $p < 0.001$, Belowground $p < 0.001$, Leaf number Belowground $p < 0.001$, Height $p < 0.001$, Root-

shoot $p < 0.001$, Nodule Number $p < 0.001$, MANOVA $p < 0.001$). While there were several significant terms, across all models there were multiple significant interactions, particularly involving the contemporary watering treatments, making their effects difficult to parse. Therefore, I constructed additional mixed-effects models that were the same structure as the previous models, but split the data into the contemporary wet and contemporary dry treatments (Table C.2). As I am primarily interested in adaptation, examining models specific to each of these contemporary watering environments will allow us to evaluate specifically adaptation to either the wet or the dry environment.

Contemporary Wet Environment: Plant Traits

Under contemporary wet conditions, while there were no significant main effects of the historical selective treatments on any of the plant traits, nor the suite of traits as a whole, there were however a complex series of significant interaction terms, that varied depending on the trait examined, involving various combinations of all 3 terms (Table C.2). Both aboveground biomass and height were significantly impacted by the interaction between the historical nitrogen and plant selective treatment; this interaction term also significantly impacted the suite of traits in the MANOVA (Aboveground $p = 0.021$, Height $p = 0.014$; MANOVA $p = 0.042$; Table C.2; Figure 4.1). Fisher's LSD *post hoc* tests suggested the addition of nitrogen when a plant was present selected for rhizobia with significantly less benefit to plant aboveground biomass and height compared to those under the control conditions. Moreover, this negative effect of rhizobia on these plant traits was only present when the rhizobia evolved with a plant, as there was no significant difference in biomass and height between nitrogen treatments if the rhizobia evolved without a plant. Plant leaf number was similarly impacted by two significant interactions, plant

history, and water history interaction ($p = 0.039$), as well as the nitrogen history and water history interactions ($p = 0.027$). *Post hoc* tests showed similar trends as the other two traits, with the nitrogen treatment degrading leaf production only when evolved with a plant (Figure 4.1). Moreover, leaf number was highest specifically when plants were inoculated with rhizobia that evolved with a plant under wet conditions, with significantly lower leaf numbers with rhizobia that had evolved with plants under dry conditions. Similar trends appeared in the *post hoc* tests for height and aboveground biomass, evidenced additionally by these traits being marginally impacted by the historical water by nitrogen interaction (Height $p = 0.060$; Aboveground $p = 0.051$), as well as plant height and the MANOVA being significantly impacted by the 3-way interaction (Height $p = 0.002$; MANOVA $p = 0.006$). Belowground biomass was significantly impacted by the interaction between the historical water and plant treatments ($p = 0.047$), with post hoc tests showing that plants inoculated with rhizobia that evolved under wet and high nitrogen conditions, with a plant, having the lowest plant biomass. Root-shoot ratio was not significantly impacted by any terms in the model (Figure 4.3; Table C.2).

Contemporary Dry Environment: Plant Traits

Under contemporary dry conditions, there were several main effects of the historical selective treatments on these plant traits. Aboveground biomass was significantly impacted by the historical watering treatment ($p = 0.007$), with plants inoculated by rhizobia from dry treatments having higher biomass than those from wet treatments (Figure 4.2). Plant height was significantly impacted by the historical plant treatment ($p = 0.001$), with plants inoculated by rhizobia that evolved with a plant increasing plant height more than those that evolved without a plant. Similar to the contemporary wet treatment discussed above, these traits were significantly

impacted by a series of complex interaction terms, depending on the trait examined (Table C.2). Both plant height and aboveground biomass were significantly impacted by the interaction between the historic plant and historic treatment, which was also reflected in the significant MANOVA term (Height $p = 0.005$; Above $p = 0.043$; MANOVA $p = 0.005$, Figure 4.2, Table C.2). *Post hoc* tests suggest that plants had the largest biomass and height when provided with rhizobia that evolved with a plant and under dry conditions. Height specifically also appeared to be impacted by the historical nitrogen treatment, with a significant 3-way interaction ($p = 0.015$), and plants that evolved under no-nitrogen treatments were significantly taller than those that evolved under nitrogen treatment. There appeared to be little significant variation between treatments when rhizobia had evolved without a plant. These historical treatments appeared to have minimal impact on belowground biomass or leaf production. Root-shoot ratio was significantly impacted by the interaction between rhizobia historical watering and plant treatment ($p < 0.002$). *Post hoc* analyses showed plants inoculated with rhizobia that evolved under dry conditions with a plant had significantly lower root-shoot ratios than all other treatments (Figure 4.3).

Nodule production

Under contemporary dry treatment, there was no significant difference between selective treatments on nodule production (Figure C.3, Table C2). There was however, under contemporary wet conditions, a significant effect of the historic water by nitrogen interaction ($p < 0.03$; Table C.2; Figure C.3), and a marginally significant effect of the historic water by plant interaction ($p < 0.08$). Correlations between nodule production and the composite plant fitness measure did vary between these selective treatments (Figure 4.4; Table C.3). Namely, under the

contemporary wet treatment, there was a significant interaction between the nodule number and the historic watering conditions in driving the correlation between nodule number and plant fitness. Specifically, while rhizobia that evolved under wet treatments resulted in a positive correlation between nodule number and plant fitness, this correlation was neutral for rhizobia that evolved under dry conditions. Somewhat similar results were observed in the contemporary dry treatments, with a significant interaction between nodule number, the rhizobia's historic watering and plant environments. I specifically found a strong positive correlation between rhizobia and plant fitness when rhizobia had evolved with a plant under droughted conditions, while all other rhizobia treatments had a more neutral relationship between plant and rhizobia fitness.

DISCUSSION

I generally found that rhizobia maximally increased plant growth when plants were paired with rhizobia that had evolved with a plant and under moisture conditions that matched that plant's contemporary moisture environment. For example, under contemporary wet conditions, plants were larger, taller, and produced more leaves when inoculated with rhizobia that had evolved with a plant under wet and low nitrogen conditions than those that evolved with a plant under dry conditions. Conversely, under contemporary dry conditions, plants were larger and taller when inoculated with rhizobia that had evolved with a plant under dry conditions than those that evolved with a plant under wet conditions, while also invested more into their aboveground tissues instead of their roots, suggesting they may have been less drought stressed. Importantly, across both contemporary wet and dry conditions, I found there was generally little variation in plant growth measures between the treatments of plants that were inoculated with rhizobia that

did not evolve with a plant. These results suggest that selection from the plant drove the evolution of rhizobia beneficial to the plant in their current moisture environment.

I note that while these beneficial rhizobia evolved when interacting with a plant, the inclusion of a nitrogen treatment drove the evolution of rhizobia with less beneficial impacts on the plant, specifically under the contemporary wet treatments. These results are consistent with prior work, which found that nitrogen addition can degrade legume-rhizobia symbioses, though I highlight that my work found the degradation of the symbiosis in significantly fewer plant generations (Weese et al. 2015). Moreover, given that nitrogen addition did not appear to influence rhizobia evolution without the presence of a plant, this suggests that nitrogen's presence may be altering selection from the plant on these rhizobia traits. These results may be the product of nitrogen addition altering the relative costs of the mutualism, which may drive these results by modifying plant investment into this mutualism. While nitrogen fixation is a costly trait for rhizobia, nitrogen fixation and thus the legume-rhizobia symbiosis may be maintained via plant investment into the mutualism, namely through selective mechanisms such as partner choice and sanctions, with plants preferentially associating and investing more resources into high-quality partners (Heath and Tiffin 2009, Westhoek et al. 2017, Oono et al. 2020, Montoya et al. 2023). However, under high nitrogen conditions, plant selection on their rhizobia may decrease, as the plants acquire their nitrogen from the environment, with plants becoming less "choosey" in with which rhizobia they associate, thus allowing for less effective partners to proliferate.

While the nitrogen addition drove the evolution of rhizobia with lower quality benefits to the plants when evolved under wet conditions, this did not appear to the same extent when these rhizobia evolved in the dry environment. Indeed, in the contemporary dry environment, plants

were generally the largest when inoculated with rhizobia that evolved with a plant under dry conditions, though granted there was a small difference in both plant biomass and height if these rhizobia evolved under dry and nitrogen conditions. However, this decrease from nitrogen was nowhere near similar to the nitrogen effect in the wet treatments. Moreover, the strong positive correlation between rhizobia and plant fitness in these same treatments under contemporary dry conditions may suggest that selection is maintaining an alignment between their fitness, selecting for rhizobia that are effective partners in dry conditions. These results may suggest that when these rhizobia evolved in droughted environments, the nitrogen addition does not alter plant selection on the rhizobia to the same degree as under the wet environment.

While rhizobia are typically viewed as benefiting the plant through nitrogen fixation, this work suggests that there was selection for rhizobia with traits beneficial to plants in their contemporary moisture environment. What rhizobia traits are the moisture and plant environments selecting upon that are underpinning this rhizobia-mediated moisture adaptation? One hypothesis may be that there may be selection for rhizobia that promote plant growth in those specific moisture environments through mechanisms not associated with nitrogen fixation. For example, various rhizosphere bacteria, including rhizobia, can facilitate plant drought tolerance through the production of plant hormones or EPS (Spaepen et al. 2009, Nett et al. 2022). Preferential allocation of plant resources to rhizobia with these traits under droughted conditions could consequently drive the evolution of these rhizobia with locally adaptive benefits to their host.

An alternative hypothesis may be that the observed rhizobia-mediated local moisture adaptation is the result of rhizobia evolving effective nitrogen fixation in the specific moisture conditions. These rhizobia's nitrogen fixation capacity may be dependent on the moisture

environment, with variation in nitrogen fixation efficiency under different moisture environments. For example, nitrogen fixation is driven by nitrogenase enzymes, which can be highly sensitive to various forms of stress in the bacteria's cellular environment (Tripathi et al. 2002, Luo et al. 2019). Consequently, under droughted conditions, various stresses, from the inherent stress of the environment to changes in the plant's internal biochemistry, may decrease nitrogenase efficiency and thus nitrogen output. Multiple generations of drought stress however may have selected for increased nitrogen fixation efficiency under droughted conditions. Effective nitrogen fixation under droughted conditions could facilitate drought tolerance as increased nitrogen availability to plants under drought can alleviate this stress by increasing water use efficiency and maintaining the synthesis of important plant hormones and antioxidants, (Guo et al. 2010, Song et al. 2019, Zhang et al. 2021). As plant partner choice mechanisms may be driven specifically through allocating increased resources to rhizobia that provide the plant with more nitrogen, this may be an effective mechanism for the evolution of rhizobia with efficient nitrogen fixation across a variety of plant environments. Consequently, under droughted conditions, selection from the plant for effective nitrogen rhizobia fixers may then facilitate plant adaptation to the droughted environment through their rhizobia partner. Given this study is the first of its kind to show that rhizobia can evolve locally adaptive benefits to their hosts outside of an explicitly nitrogen-related symbiosis, to my knowledge, future work should thoroughly follow up on these results, investigating the specific mechanisms driving this rhizobia-mediated adaptation.

While the differing effects on plant fitness and phenotype between rhizobia sourced from the different selection treatments highlight that these treatments have driven significant evolution of the rhizobia populations, the drivers of this rhizobia evolution are unclear. As I started the

evolution experiment with 28 strains of rhizobia, this evolution could have been driven primarily by selection on the standing variation in the population, altering the relative frequencies of each of the strains between treatments. Consequently, differences in the observed treatments in their effects on plants may have just been changes in which strains were isolated at the end of the selection phase. Granted however, my prior work with these same 28 strains showed little capacity for mediating drought adaptation, with strong correlations between rhizobia quality in wet vs. dry conditions (Ricks, unpublished data), perhaps making it unlikely that such changes could have been driven by selection on standing variation alone. Therefore, this evolution may alternatively have been driven by *de novo* mutations in the rhizobia genome that produced adaptive rhizobia traits and were then selected upon, driving the observed differences in plant phenotype between treatments. For this to occur, there needs to be sufficient rhizobia generations for these mutations to accumulate, be selected upon, and reach a high enough frequency in the population that I could have sampled from the population. With only 4 plant generations, it may seem unlikely that there would have been sufficient time for such mutations to drive the observed phenotypic changes in this experiment. However, rhizobia reproduction is not tied solely to that of the plant. Free-living rhizobia in the soil may have significantly faster generations than those in the plant, which may then act as reservoirs for genetic diversity through horizontal gene transfer. I anticipate the future sequencing of these strains can be used to elucidate the driving factors of these populations' evolution.

CONCLUSION

This work suggests that the plant, moisture, and nitrogen environments interactively select upon rhizobia bacteria, specifically with respect to the rhizobia traits associated with plant adaptation.

In particular, the evolution of rhizobia interactions with their plant host appears to be driven by selection from the plant; in the absence of plant selection, the abiotic environments had little impact on the evolution of the rhizobia's plant effect. Importantly, these plants appear to be drivers of the evolution of rhizobia with benefits adaptive to the plant's current environment. While the potential mechanisms through which rhizobia might be evolving locally adaptive plant phenotypes remain unclear, these certainly remain tantalizing hypotheses for future sequencing and experimental work. Regardless, however, this work suggests that the rhizobia symbiosis does not collapse in the face of stress, but rather, this symbiosis can rapidly adapt to its current environment, serving as an engine for host local adaptation. Whether these results can be translated to other host-microbe interactions is unclear, as unlike many other symbioses, the legume-rhizobia symbiosis is highly specialized, and the result of millions of years of coevolution. Future work should investigate similar questions across a variety of systems and environments. Continuing to study the drivers of symbiont evolution, specifically with respect to their traits associated with host adaptation, will facilitate future insights into host fitness phenotypes, and patterns of adaptation.

FIGURES AND TABLE

Figure 4.1: Various plant traits from our greenhouse experiment from plants grown under contemporary wet conditions, including above- (a) and belowground biomass (b), leaf number (c), and height (d). Data are grouped by the inoculated rhizobia's evolutionary history: the historical plant treatment (with or without a plant; within each figure, these are displayed on the left and right panels), the historical moisture treatment (wet or dry; delineated by blue vs. orange coloring respectively), and the historical nitrogen treatment (control of nitrogen addition; delineated by closed vs. open circles respectively). I present here estimated marginal means, with bars representing 95% confidence intervals generated from the standard error. Lettering represents *post hoc* Fisher's LSD, with groups that differ in their lettering being significantly different from one another. I call the reader's attention to the fact that the y-axes do not begin at zero, in order to allow for easier comparison between groups.

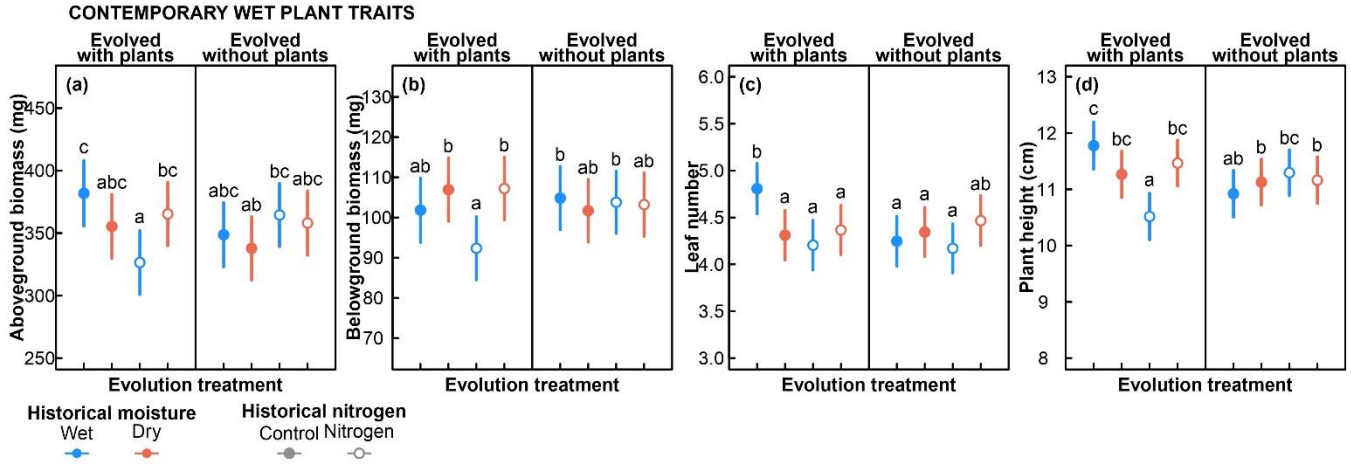


Figure 4.2: Various plant traits from our greenhouse experiment from plants grown under contemporary dry conditions, including above- (a) and belowground biomass (b), leaf number (c), and height (d). Data are grouped by the inoculated rhizobia's evolutionary history: the historical plant treatment (with or without a plant; within each figure, these are displayed on the left and right panels), the historical moisture treatment (wet or dry; delineated by blue vs. orange coloring respectively), and the historical nitrogen treatment (control of nitrogen addition; delineated by closed vs. open circles respectively). I present here estimated marginal means, with bars representing 95% confidence intervals generated from the standard error. Lettering represents *post hoc* Fisher's LSD, with groups that differ in their lettering being significantly different from one another. I call the reader's attention to the fact that the y-axes do not begin at zero, in order to allow for easier comparison between groups.

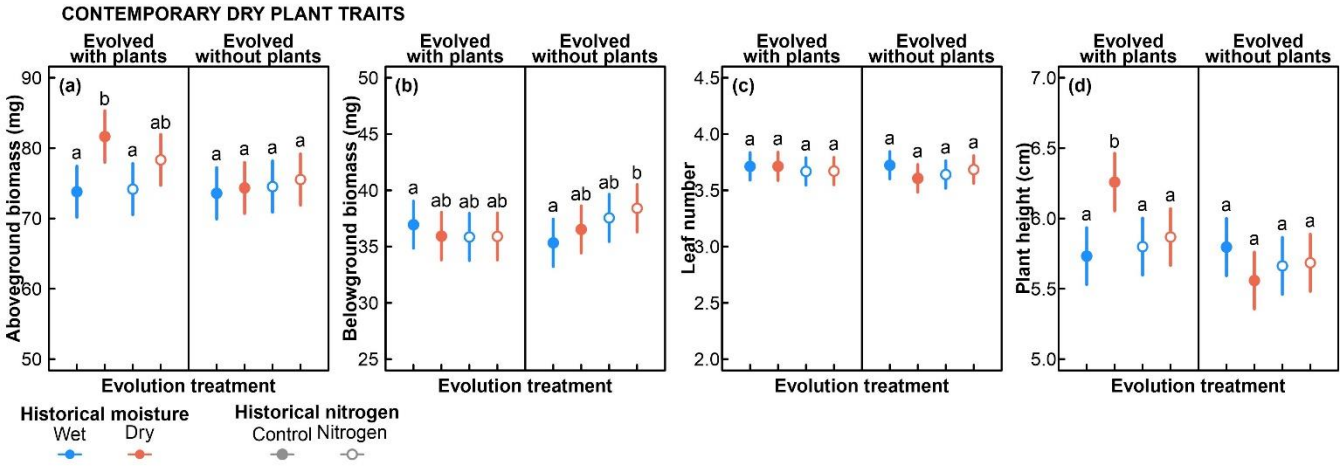


Figure 4.3: Root-shoot ratio data from our greenhouse experiment from plants grown, under both contemporary wet (a) and contemporary dry (b) conditions. Data are grouped by the inoculated rhizobia's evolutionary history: the historical plant treatment (with or without a plant; within each figure, these are displayed on the left and right panels), the historical moisture treatment (wet or dry; delineated by blue vs. orange coloring respectively), and the historical nitrogen treatment (control of nitrogen addition; delineated by closed vs. open circles respectively). I present here estimated marginal means, with bars representing 95% confidence intervals generated from the standard error. Lettering represents *post hoc* Fisher's LSD, with groups that differ in their lettering being significantly different from one another.

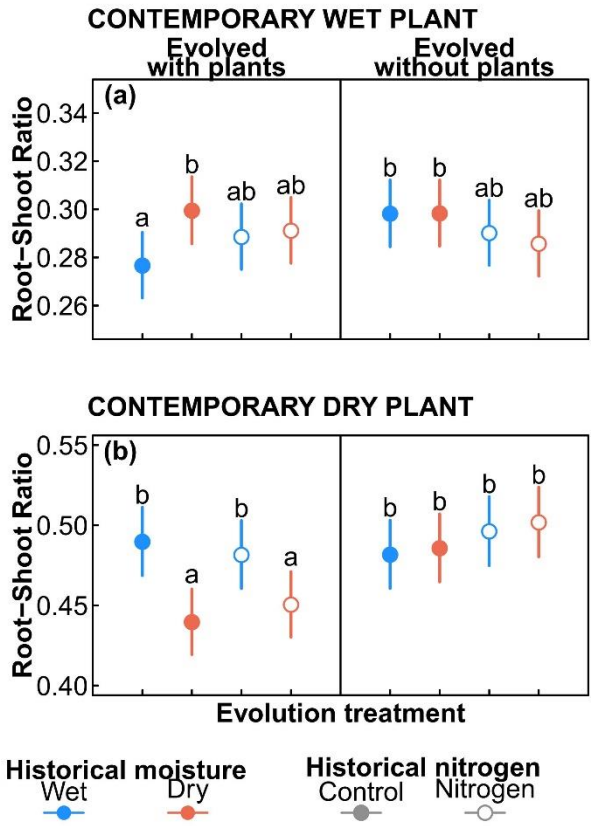


Figure 4.4: Correlations between estimated plant and rhizobia fitness. Plant fitness is the first PC axis combining plant height, leaf number, and aboveground biomass (see Table C.1 for loadings), and rhizobia nodule number as a proxy for rhizobia fitness. We specifically display residuals for these estimates, after taking account of block, herbivory, and the nodule counter for the nodule data. We separate these data based on the contemporary watering environment. Within each of these treatments, we specifically display the significant main effects from our model (see Table C.3), for which variables determine this correlation. Namely for the contemporary wet treatment, this was determined by the rhizobia's historical moisture environment, while for the contemporary dry treatment, this was determined by the interaction between the rhizobia's historical moisture and plant environment.

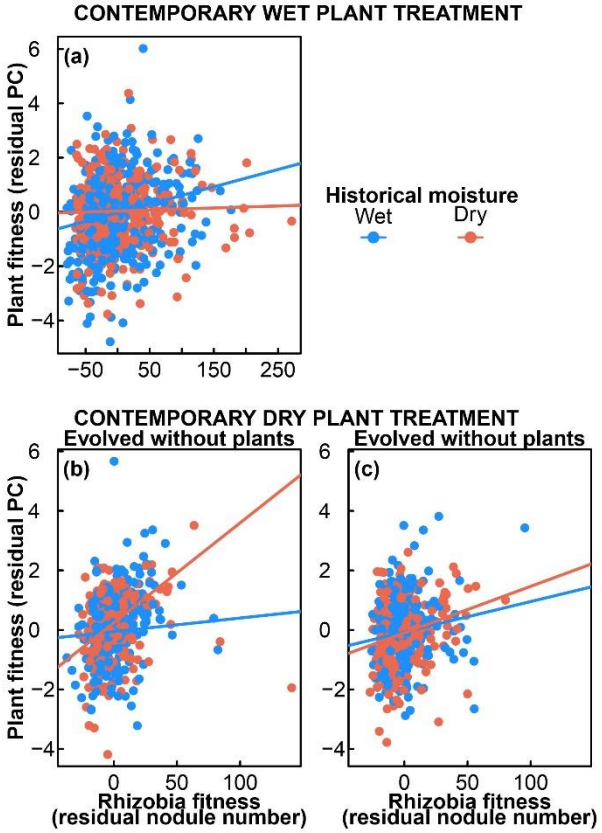


Table 4.1: Mixed model ANOVAs for the effects of the contemporary watering treatment (wet vs. dry), historic watering treatment (wet vs. dry), historic plant treatment (present vs. absent), historic nitrogen treatment (control vs. nitrogen), and their interactions on multiple traits associated with plant health, including above- and belowground biomass, leaf number, as well as height. We additionally show a MANOVA that combines all 4 plant traits together. We additionally display two auxiliary traits, displayed on the second half of this table, namely root-shoot ratio and nodule number. We display the F value for each model term. Terms with an associated *p* value less than 0.05 are bolded. + $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Plant Traits						
Model Term	**df**	**Aboveground**	**Belowground**	**Height**	**Leaves**	**MANOVA**
Contemporary Water	1	**785.27*** **	**247.51*** **	**483.50*** **	**75.20*** **	**1512.17*** **
Historic Water	1	0.03	1.76	1.69	0.00	1.00
Historic Plant	1	0.54	0.57	**5.17***	2.11	**4.92*** **
Historic Nitrogen	1	0.05	0.22	2.22	1.94	1.72
ContWater x HistWater	1	0.28	1.41	0.01	0.05	1.49
ContWater x HistPlant	1	0.02	0.05	0.39	0.54	0.65
HistWater x HistPlant	1	1.15	2.98^{+}	3.30^{+}	2.49	3.26*
ContWater xHistNitro	1	0.02	0.83	0.26	0.86	0.79
HIstWater x HistNitro	1	3.36^{+}	1.22	1.96	**5.74***	1.95^{+}
HistPlant x HistNitro	1	**5.72***	1.61	**7.34** **	2.78^{+}	**2.39***
ContWater x HistWater x HistPlant	1	0.33	**5.22***	0.40	**4.08***	**5.63*** **
ContWater x HistWater x HistNitro	1	3.76^{+}	0.85	3.76^{+}	2.30	1.69
ContWater x HistPlant x HistNitro	1	**4.48***	0.12	3.10^{+}	1.55	2.15^{+}
HistWater x HistPlant x HistNitro	1	2.07	0.45	2.61	0.39	1.27
ContWater x HistWater x HistPlant x HistNitro	1	2.84^{+}	0.22	**14.10*** **	2.15	**5.41*** **
Auxiliary Traits						
Model Term	**df**	**Root-Shoot Ratio**	**Nodule Number**			
Contemporary Water	1	**128.65*** **	**815.49*** **			
Historic Water	1	1.41	0.01			
Historic Plant	1	**10.22** **	0.27			
Historic Nitrogen	1	0.7	0.01			
ContWater x HistWater	1	**6.33***	0.12			
ContWater x HistPlant	1	**4.56***	0.95			
HistWater x HistPlant	1	1.86	3.68^{+}			
ContWater xHistNitro	1	1.82	0.62			
HIstWater x HistNitro	1	0.11	2.91^{+}			
HistPlant x HistNitro	1	0.01	0.00			
ContWater x HistWater x HistPlant	1	**10.43** **	1.59			
ContWater x HistWater x HistNitro	1	1.72	**4.16***			
ContWater x HistPlant x HistNitro	1	2.28	0.78			
HistWater x HistPlant x HistNitro	1	0.00	0.72			
ContWater x HistWater x HistPlant x HistNitro	1	0.85	0.60			

CHAPTER 5: PERSPECTIVES AND CONCLUSIONS

While interactions between hosts and their associated microbes can significantly contribute to host phenotype, these interactions are often cryptic, with spatially and temporally variable effects on the host. In this dissertation I attempted to elucidate the determinants of these host-microbe interactions by taking an evolutionary perspective on these interactions and evaluating when interactions with an adaptive benefit to the host may be selected for. I specifically used the microbes associated with plants as a model system, considering the evolution of both the plants as well as microbes, and the impact of this interaction's evolution on the host plant's phenotype. In my first chapter, I reviewed coevolutionary interactions between plants and their associated microbes, identifying gaps in the literature. To determine the selective drivers of interactions with an adaptive benefit to the host plant, in the following three chapters I experimentally characterized these interactions across a variety of environments, evaluating when they provided locally adaptive benefits to their hosts. Broadly, I found that these plant-microbe interactions could quickly evolve to provide locally adaptive benefits to the plants, though the drivers of this local adaptation varied.

In my second chapter, I evaluated the impact of plant evolution on their interactions with their root-associated microbes in facilitating plant local adaptation by collecting genotypes of *Bromus tectorum*, an annual grass, from a variety of salinity habitats. These salinity habitats represented a natural evolution experiment, as I assumed that these populations adapted to their home salinity conditions, thus allowing me to assess how these plant populations' interactions with their associated microbes had evolved with respect to their impact on plant local adaptation. In growing these populations in the greenhouse under variable salinity and microbial environments, I found that patterns of plant local adaptation to their home salinity only emerged

when provided with a microbial inoculum with which to interact. This result suggested that interactions with these microbes may have been essential in the plant's adaptation to the environment. Specifically, I hypothesized that plants had evolved to adaptively interact with these microbes in their home salinity environment, facilitating the observed patterns of plant local adaptation.

In both my third and fourth chapters, I examined the drivers of microbial evolution, leveraging the rapid generation times of microbes to use an experimental evolution approach. I was principally interested in examining the role of the plant in selecting upon their associated microbes and driving the evolution of microbes that provide a locally adaptive benefit to the plant. Consequently, in both chapters I evolved microbes under variable moisture environments (wet or dry), and either with or without a plant, and then inoculated these plants back onto plants under these conditions. If plants select for locally adaptive microbes, I would predict that plants would have the highest fitness in a given moisture environment when provided with microbes that evolved in that environment and with a plant. Indeed, in my fourth chapter, wherein I evolved rhizobia, a symbiont of leguminous plants, I found rhizobia had evolved traits only when with a plant to facilitate plant adaptation to the moisture environment. This result suggested that these microbial traits had evolved to adaptively benefit the plant.

However, these results contrasted with those from the third chapter. Therein, I used a similar approach, but instead of specifically targeting rhizobia, I used whole communities of soil microbes which I inoculated onto plants by using whole soils. Principally, I found that these microbial communities again evolved to facilitate benefits locally adaptive to the plant's moisture environment, however unlike in the previous rhizobia study, the presence of the plant was not necessary in selecting for these locally adaptive communities. These results suggested that while

microbes can evolve traits to benefit their hosts, these beneficial traits can sometimes simply be a byproduct of these microbes' own adaptation to their environment.

A perhaps tantalizing subject for speculation is why I observed seemingly opposite results between these two final chapters, namely where the plant drove locally adaptive microbes in one experiment while these locally adaptive microbes emerged simply as a byproduct of adaptation to the abiotic environment in the other. I hypothesize that these differences may be a product of the nature of the microbial benefits in the specialized rhizobia system versus the generalized soil microbial communities. Indeed, rhizobia-legume interactions are highly specialized, involving a complex series of signals between the plant and microbes, with the plants preferentially rewarding beneficial microbial partners (Westhoek et al. 2017, Oono et al. 2020). This reward system maintains this symbiosis as nitrogen fixation is an energetically expensive trait that may not provide the rhizobia with significant benefits outside of those provisioned by the plant. Consequently, when not associating with a plant host, there may not be selective pressure for these rhizobia to evolve traits beneficial to their plant hosts. In contrast, in a general soil microbial community, there are a variety of microbes that provide a variety of benefits to plants, with many of these benefits only a byproduct of the microbes' function, including nutrient cycling, decomposition, etc. Consequently, adaptation of these microbes to a given environment may incidentally facilitate continued access for these plants to these microbial benefits.

Overall, this dissertation illustrates that host-microbe interactions are dynamic, likely a result of the continuous selection on both the plant and microbial traits that drive this interaction. In an effort to further uncover the drivers of host phenotype, future work may continue to investigate these interactions' evolution across a variety of host-microbe systems. I specifically

highlight that while the evolution of molecular mechanisms underlying these plant-microbe interactions was beyond the scope of this work, examining these mechanisms may be a particularly fruitful area of future work.

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APPENDIX A: SUPPLEMENTARY FIGURES AND TABLES FOR CHAPTER 2

Figure A.1: Locations of populations collected for this contribution. These data represent a total of 10 populations, 5 designated as saline and 5 designated as nonsaline. Populations from saline habitats are colored in orange and populations from nonsaline habitats are colored in green-blue. Contour lines represent 609 meters of elevation (2,000 feet).

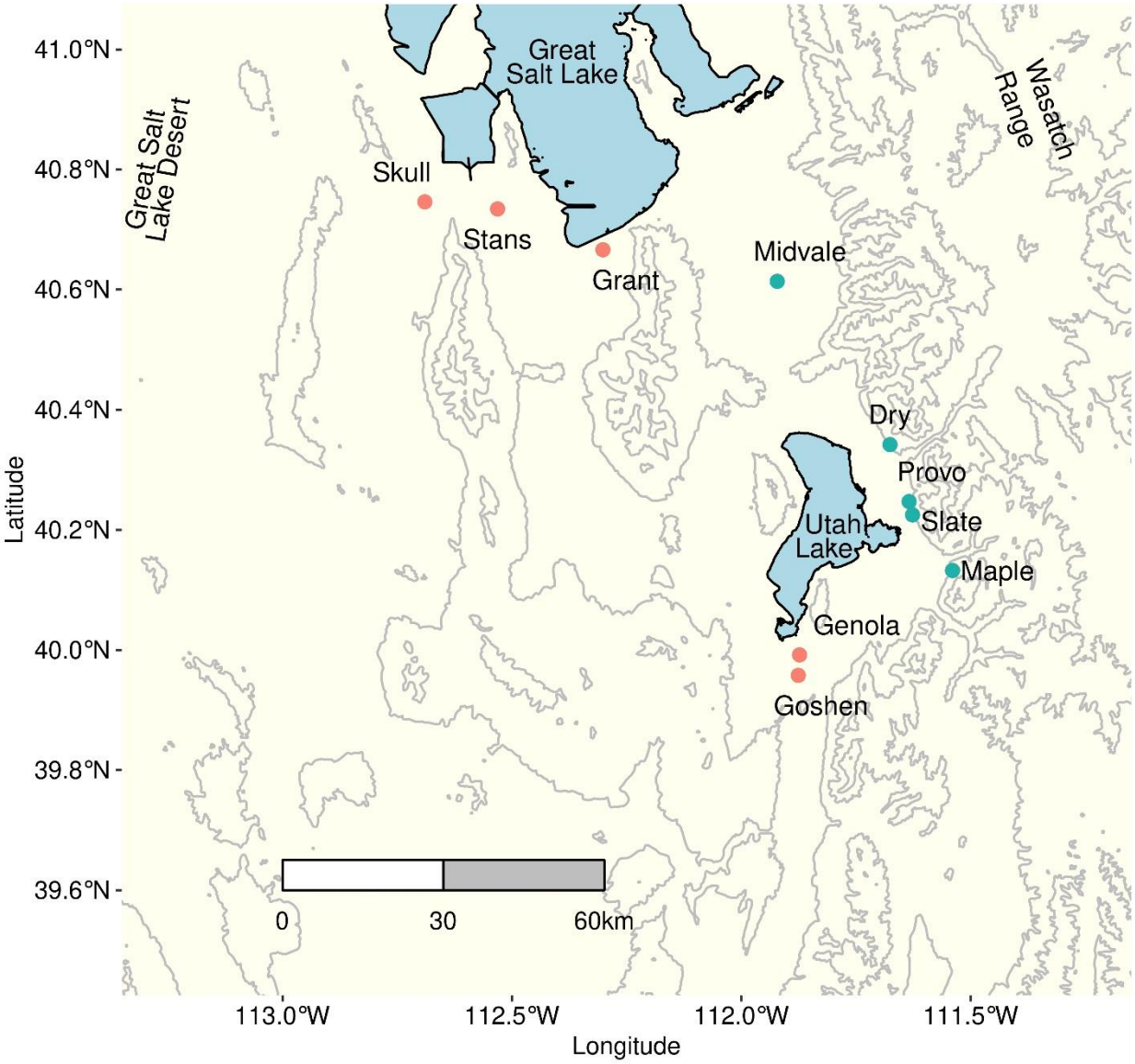

Figure A.2: Soil conductivity from soils sampled at each *Bromus tectorum* population. These data represent a total of 10 populations, 5 designated as saline and 5 designated as nonsaline. Conductivity is a proxy measure for soil salinity. Briefly, soils were diluted in a 1:5 dilution with distilled water, and shaken, after which I measured conductivity of the solution using a conductivity meter (Model 407303, Extech instruments, Nashua, New Hampshire). These measurements were adjusted by their dilution factor and compared to known standards to characterize the overall conductivity of the soil.

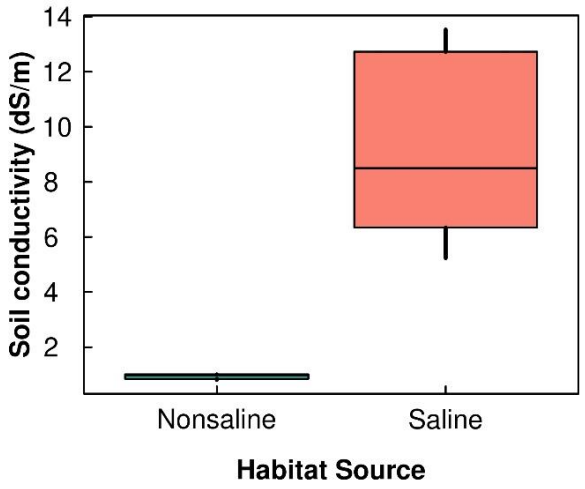

Figure A.3: Germination rate of *Bromus tectorum* in the greenhouse. As greenhouse salinity treatments did not begin until several weeks after germination, there is no differentiation between these treatments here. Data are grouped by the plant's habitat of origin (saline or nonsaline), as well as the provided microbial inoculum. Bars represent 95% confidence intervals of the mean generated from the standard error.

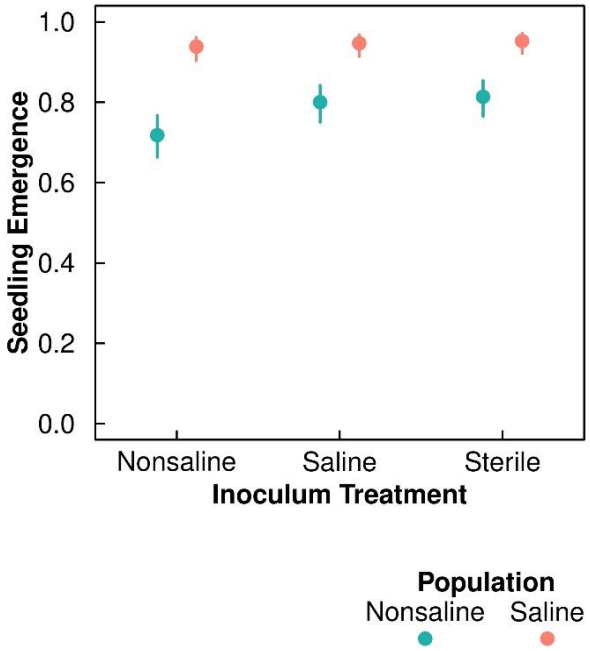

Figure A.4: Survival data from our greenhouse experiment. Data are grouped by the plant's habitat of origin (saline and nonsaline populations), greenhouse salinity treatment (saline and nonsaline), as well as the inoculum provided (saline microbes, nonsaline microbes, and sterile). I display both the data for individual populations, as well the average of populations based on their habitat of origin. Individual populations are semi-transparent, while averages of habitat are solid. I chose specific orthogonal contrasts *a priori* to evaluate salinity adaptation; namely, I compare the habitat of origin under each salinity and microbial combination, as indicated in the figure, as is appropriate to evaluate local adaptation. Bars represent 95% confidence intervals of the mean generated from the standard error. The statistical significance of each comparison is indicated using the symbology as follows: ns $p > 0.10$; + $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

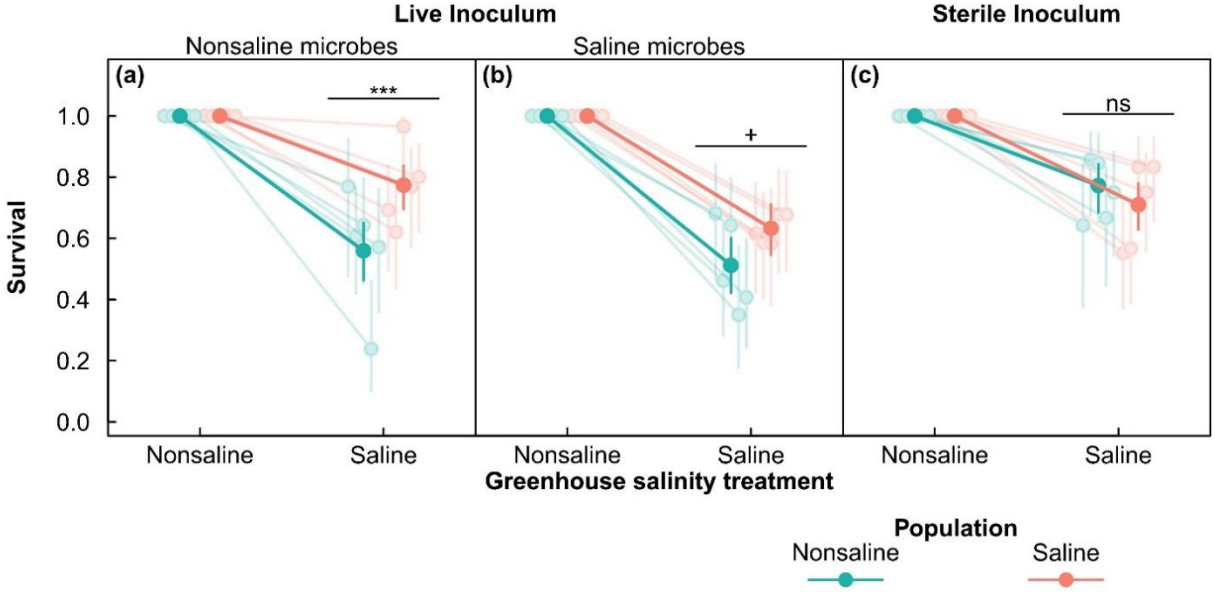

Figure A.5: Above- and belowground biomass data from our greenhouse experiment, excluding plants that died. Data are grouped by the present greenhouse salinity environment (nonsaline or saline), the plant's habitat of origin (nonsaline or saline), as well as the provided microbial inoculum (nonsaline or saline). I display both the data for individual populations, as well the average of populations based on their habitat of origin. Individual populations are semi-transparent, while averages of habitat are solid. I chose specific orthogonal contrasts *a priori* to evaluate salinity adaptation; namely, I compare the habitat of origin under each salinity and microbial combination, as indicated in the figure, as is appropriate to evaluate local adaptation. Bars represent 95% confidence intervals of the mean generated from the standard error. The statistical significance of each comparison is indicated using the symbology as follows: ns $p > 0.10$; + $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

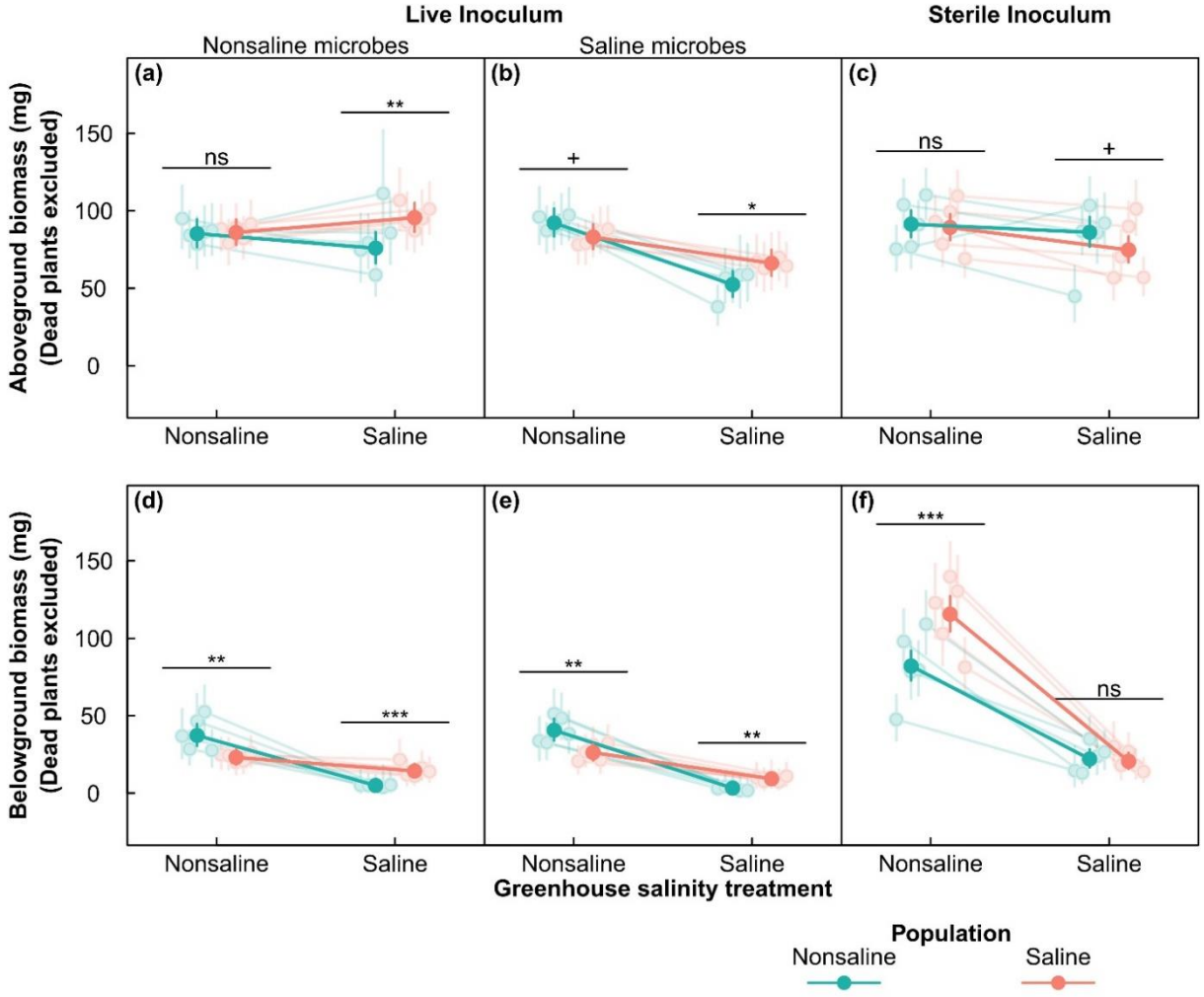

Figure A.6: Estimated fitness from aster models (previously described) as well as survival data from our greenhouse experiment for plants grown under saline conditions. Data are grouped by the provided microbial inoculum (nonsaline, saline, or sterile) as well as by the population, and correlated with the observed conductivity from these populations. Bars represent 95% confidence intervals of the mean generated from the standard error. I included a dashed red line to delineate the separation between the populations classified as 'saline' vs 'nonsaline' adapted. Populations with conductivity lower than the line were classified as 'nonsaline' while populations above the line were classified as 'saline'.

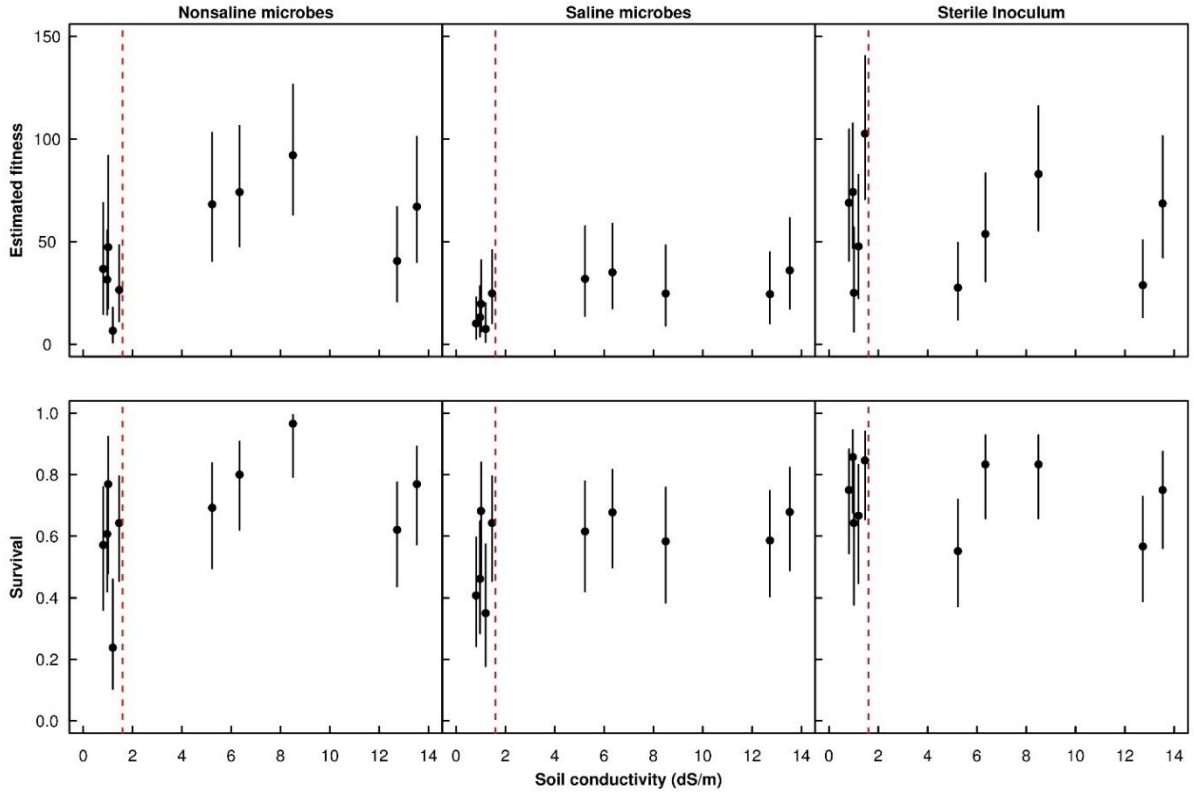

Table A.1: Locations of populations collected for this contribution.

Habitat	Population	Latitude	Longitude	Conductivity (dS/m)
Nonsaline	Maple	40.132	-111.545	1.46
Nonsaline	Provo	40.247	-111.634	1.20
Nonsaline	Midvale	40.613	-111.922	0.81
Nonsaline	Slate	40.225	-111.627	0.97
Nonsaline	Dry	40.342	-111.676	1.01
Saline	Goshen	39.957	-111.876	8.50
Saline	Genola	39.991	-111.873	12.73
Saline	Stans	40.734	-112.531	13.53
Saline	Grant	40.666	-112.302	5.23
Saline	Skull	40.746	-112.695	6.34

Table A.2: Model evaluating the effects of the plant's habitat of origin (saline and nonsaline populations), greenhouse salinity treatment (saline and nonsaline), the inoculum provided (saline microbes, nonsaline microbes, and sterile) and their interactions, on the estimated fitness (total biomass and survival, using aster models), with maternal line as a random effect. Analogous using population as a random effect can be seen in Table 2.1. Separate models were also built for the components going into this measure of estimated fitness, including above- and belowground biomass, and survival. For survival, terms including the salinity treatment were excluded, as there was no variance in survival under nonsaline treatments (100% survival). For estimated fitness, I display the deviance from the aster model for each model term. For above- and belowground biomass models I display both the F value and Mean Square for each model term. For survival, I display the Chi-Squared for each model term. Terms with an associated *p* value less than 0.05 are bolded. + $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Model Term	**df**	**Estimated Fitness (Total Biomass + Survival)**	**Aboveground**		**Belowground**		**Survival**
		Deviance	MS	F value	MS	F value	ChiSq
Habitat Source	1	**4.2856***	4.05	0.94	17.9	2.64	**5.94***
Salinity	1	**498.92*****	**165.83**	**38.58*****	**3964**	**584.47*****	NA
Inoculum	2	**102.51*****	**68.68**	**15.98*****	**1316**	**194.06*****	**13.26****
Habitat x Salinity	1	**6.7118****	**30.83**	**7.17****	**100.1**	**14.76*****	NA
Habitat x Inoculum	2	4.9339^{+}	**22.97**	**5.34****	17.1	2.52^{+}	**10.93****
Salinity x Inoculum	2	**38.901*****	**68.68**	**15.98*****	**214.4**	**31.61*****	NA
Habitat x Inoculum x Salinity	2	**28.961*****	**27.70**	**6.45****	**176.7**	**26.06****	NA

Table A.3: Results of the specific *a priori* comparisons used to evaluate local adaptation. These comparisons are separated by the various measured I used to evaluate local adaptation in the lefthand column, including the aster estimate of fitness, above and belowground biomass, and survival. In the first two rows of the table, I display the various combinations of inoculum and greenhouse salinity treatments; within each of these treatment combinations, I compared the fitness measures between nonsaline and saline populations. I display the estimate for these measures for the nonsaline and saline populations as well as the *p* value associated with the comparison between those two estimates. I note that these comparisons are displayed in Figures 2.2, A.4 & A.5

	Inoculum	Nonsaline inoculum	Nonsaline inoculum	Saline inoculum	Saline inoculum	Sterile inoculum	Sterile inoculum
	Greenhouse salinity	Nonsaline greenhouse	Saline greenhouse	Nonsaline greenhouse	Saline greenhouse	Nonsaline greenhouse	Saline greenhouse
	Habitat Source						
Estimated Fitness (Total biomass + survival, mg)	Nonsaline populations	122.62	26.39	135.75	14.72	177.11	66.04
	Saline populations	110.55	67.35	111.17	30.44	208.92	49.90
	P	0.233	<0.001	0.012	0.006	0.164	0.359
Aboveground (mg)	Nonsaline populations	85.6	75.7	92.4	51.9	91.6	86.6
	Saline populations	86.0	95.6	83.0	66.0	89.3	74.9
	P	0.902	0.006	0.134	0.023	0.719	0.078
Belowground (mg)	Nonsaline populations	37.31	4.99	40.99	3.23	82.29	22.20
	Saline populations	22.90	14.19	26.12	9.16	115.28	20.44
	P	0.001	<0.001	0.001	0.009	<0.001	0.714
Survival	Nonsaline populations	0.99	0.56	1.00	0.51	1.000	0.77
	Saline populations	1.00	0.77	1.00	0.63	1.000	0.71
	P	NA	<0.001	NA	0.057	NA	0.263

APPENDIX B: SUPPLEMENTARY FIGURES AND TABLES FOR CHAPTER 3

Figure B.1: I assessed potential nutritional differences between the various inoculums, specifically examining soil inorganic N. Data are grouped by the microbial inoculums historic watering treatment (wet or dry), and the microbial inoculum's historic plant treatment (*Arabidopsis thaliana* or *Brassica rapa* or no plant). Bars represent 95% confidence intervals of the mean generated from the standard error.

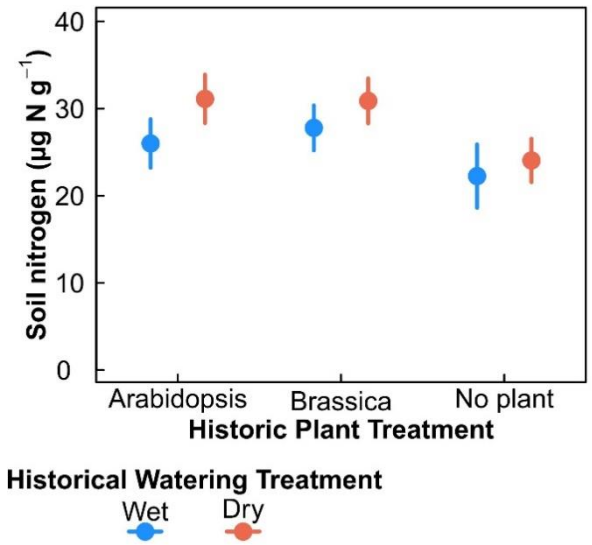

Figure B.2: To examine if variation in nitrogen content between different inoculums (see supplemental figure 1) influenced the inoculated plant's aboveground biomass, I correlated the two variables. I created a model for both contemporary watering treatments (wet or dry). To control for variation between treatments, I extracted the residuals from the models described in Table 1 and regressed those against residual nitrogen content.

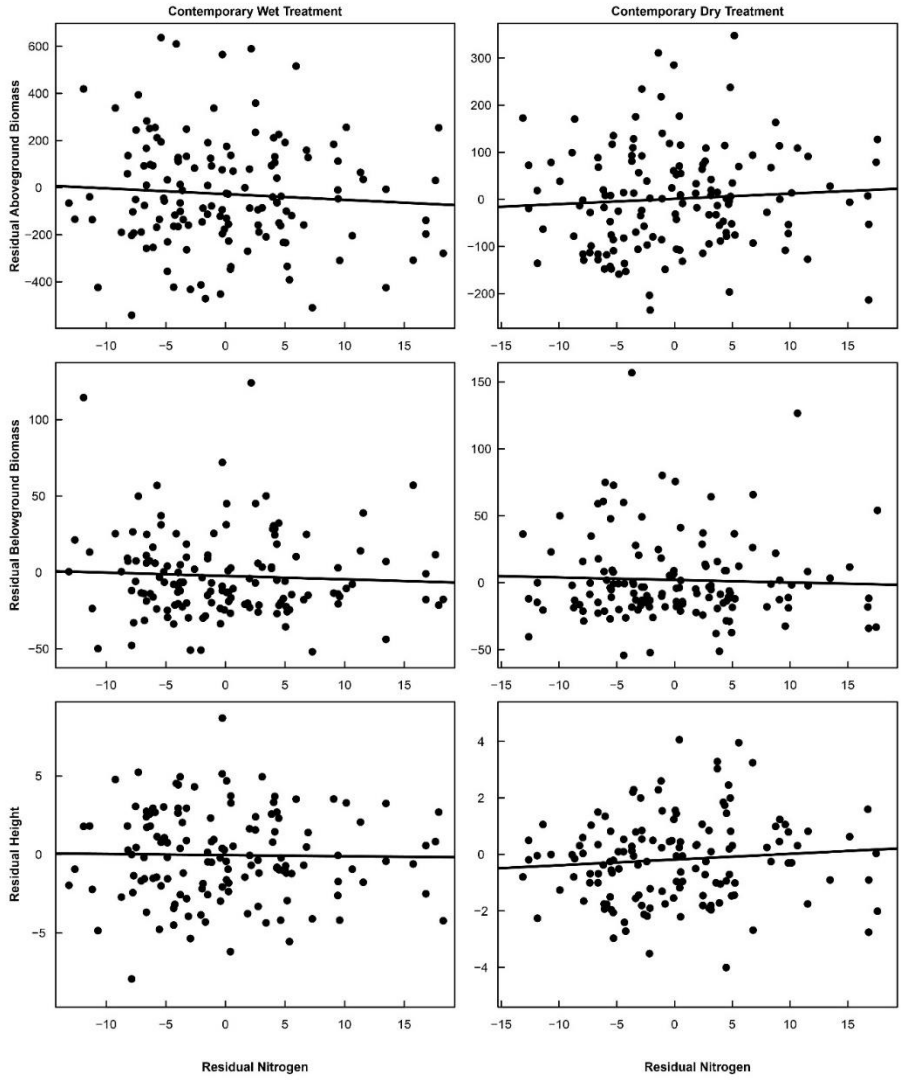

Table B.1: Mixed model ANOVAs for the effects of the historic plant conditioning treatment (*Arabidopsis thaliana* vs. *Brassica rapa* vs. no plant), historic watering treatment (wet vs. dry), the microbial status (live vs. sterile), and their interactions on multiple plant traits, including above- and belowground biomass, as well as height. I additionally show a MANOVA that combines all 3 plant traits together. This table compares to the models presented in Table 1, but instead includes the results from the separate models for plants under the contemporary wet treatment and those under the contemporary dry treatment. I display both the F value for each model term. Terms with an associated *p* value less than 0.05 are bolded. + $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

		Contemporary Wet					**Contemporary Dry**			
Model Term	**df**	**Aboveground Biomass**	**Belowground Biomass**	**Plant Height**	**MANOVA**		**Aboveground Biomass**	**Belowground Biomass**	**Plant Height**	**MANOVA**
Historic Water	1	**14.60*****	**16.89*****	**10.22****	**6.02*****		**32.00*****	**11.99*****	**8.02****	**12.82*****
Historic Plant	2	2.61^{+}	1.63	2.47^{+}	1.84+		0.43	2.16	0.10	1.78
Microbe	1	**33.07*****	**13.46*****	**30.39*****	**13.50*****		**40.53*****	**34.47*****	**8.81****	**20.68*****
HistWater x HistPlant	2	**7.49*****	**4.02***	**3.18***	**3.50****		**5.17****	0.47	2.51+	**4.38*****
HistWater x Microbe	1	**4.48***	**7.12****	**14.04*****	**5.25****		**39.57*****	**31.33*****	**21.74*****	**17.30*****
HistPlant x Microbe	2	1.84	2.96^{+}	0.09	1.59		1.98	1.19	0.36	1.12
HistPlant x HistWater x Microbe	2	0.06	0.25	1.17	0.95		1.40	2.70^{+}	0.98	1.72

Table B.2: Mixed model ANOVAs for the effects of the historic plant conditioning treatment (*Arabidopsis thaliana* vs. *Brassica rapa* vs. no plant), the historic watering treatment (wet vs. dry), their interactions on the microbial effect on multiple plant traits, including above- and belowground biomass, as well as height. I additionally show a MANOVA that combines all 3 plant traits together. Note, that this these models contrast from the previous models as there is no 'Microbe' term as the response variable here was the effects of the microbes on biomass (the ratio of plant biomass provided with live soils vs. plant biomass provided with sterile soils). This table compares to the models presented in Table 2, but instead includes the results from the separate models for plants under the contemporary wet treatment and those under the contemporary dry treatment. I display both the F value for each model term. Terms with an associated *p* value less than 0.05 are bolded. + $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

		Contemporary Wet					**Contemporary Dry**			
Model Term	**df**	**Aboveground Biomass**	**Belowground Biomass**	**Plant Height**	**MANOVA**		**Aboveground Biomass**	**Belowground Biomass**	**Plant Height**	**MANOVA**
Historic Water	1	**11.22*****	**12.71*****	**19.06*****	**6.92*****		**32.29*****	**21.76*****	**21.03*****	**9.32*****
Historic Plant	2	1.67	0.54	0.17	0.67		1.03	0.70	0.21	0.68
HistWater x HistPlant	2	0.40	0.53	1.50	0.84		0.94	2.64	0.99	1.14

Table B.3: Parameters for models describing soil microbial respiration across a soil moisture gradient. Parameters are given as the estimates of the 95% confidence interval for each parameter. Separate models were constrcuted for the two historical soil treatments: soil microbes from the historic dry treatments and soil microbes from the historic wet treatments. Models were fit using a maximum likelihood models methods to a biologically relevant function, first described by Lennon *et al.* (2012). The model can be described as:

$$R = R_{Max}\left(\exp\left[-\left|\frac{W-W_{Opt}}{\sigma}\right|^{\tau}\right]\right)$$

where R is the predicted respiration rate; R_{Max} is the max respiration rate of the curve; W is soil water content; W_{Opt} is the soil water content corresponding to the maximum respiration rate; σ is the rate that respiration declines as the soil moisture moves away from W_{Opt}; and τ is the kernel that defines the general shape of the response curve.

Term	Historic Wet Microbes	Historic Dry Microbes
R_{Max}	0.516–0.540	0.538–0.562
W_{Opt}	0.206–0.210	0.195–0.199
σ	0.080–0.088	0.088–0.098
τ	1.285–1.401	1.519–1.632

APPENDIX C: SUPPLEMENTARY FIGURES AND TABLES FOR CHAPTER 4

Figure C.1: Comparison of various traits from our greenhouse experiment between plants inoculated with live rhizobia vs sterile inocula, under both contemporary wet and contemporary dry conditions. We include above- and belowground biomass, leaf number, plant height, and nodule number. We present here violin plots, displaying the density distribution of the data, with boxplots and means in the center.

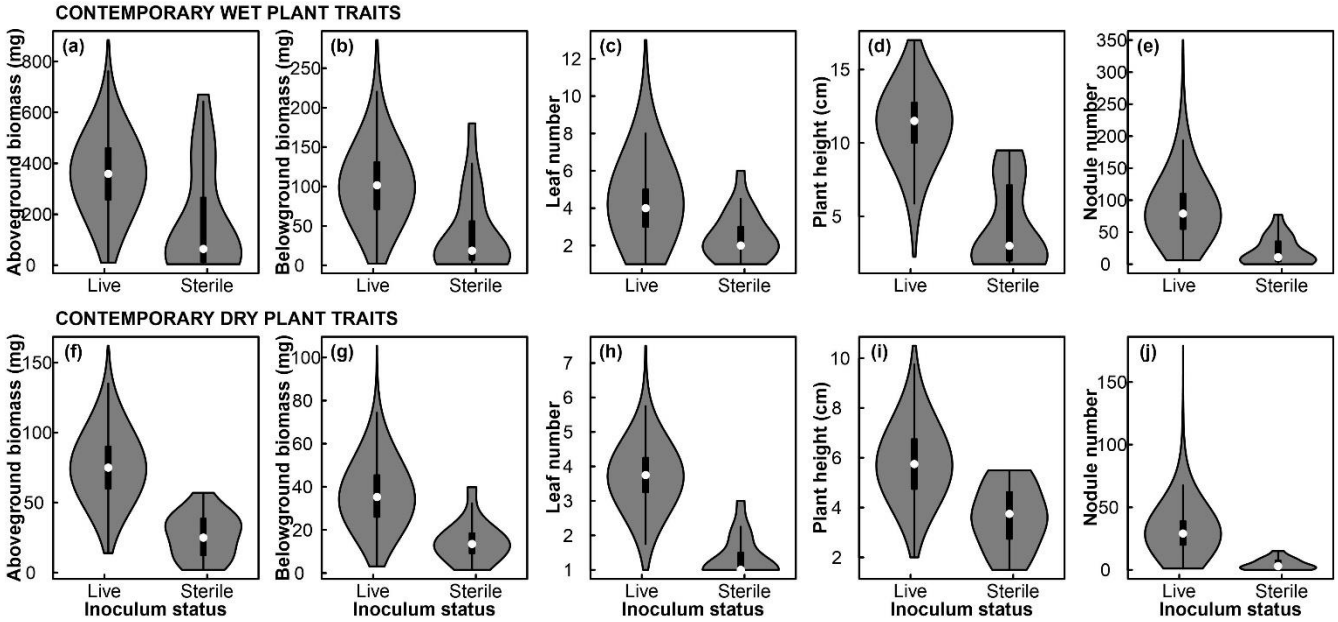

Figure C.2: Correlation between nodule number on a root system to the total nodule mass on the root system. Data come from a random subset of 174 plants from the contemporary dry conditions.

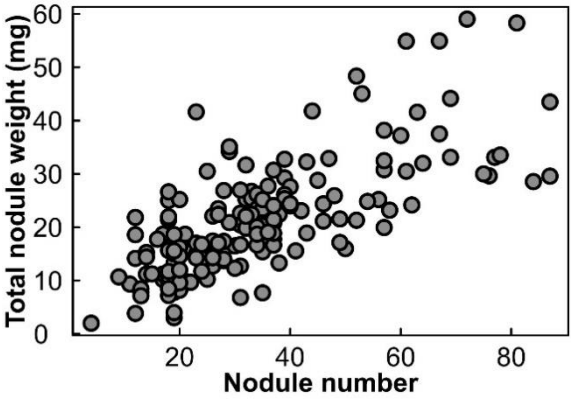

Figure C.3: Nodule number from our greenhouse experiment from plants grown, under both contemporary wet (a) and contemporary dry (b) conditions.. Data are grouped by the inoculated rhizobia's evolutionary history: the historical plant treatment (with or without a plant; within each figure, these are displayed on the left and right panels), the historical moisture treatment (wet or dry; delineated by blue vs. orange coloring respectively), and the historical nitrogen treatment (control of nitrogen addition; delineated by closed vs. open circles respectively). We present here estimated marginal means, with bars representing 95% confidence intervals generated from the standard error. Lettering representing *post hoc* Fisher's LSD, with groups that differ in their lettering being significantly different from one another.

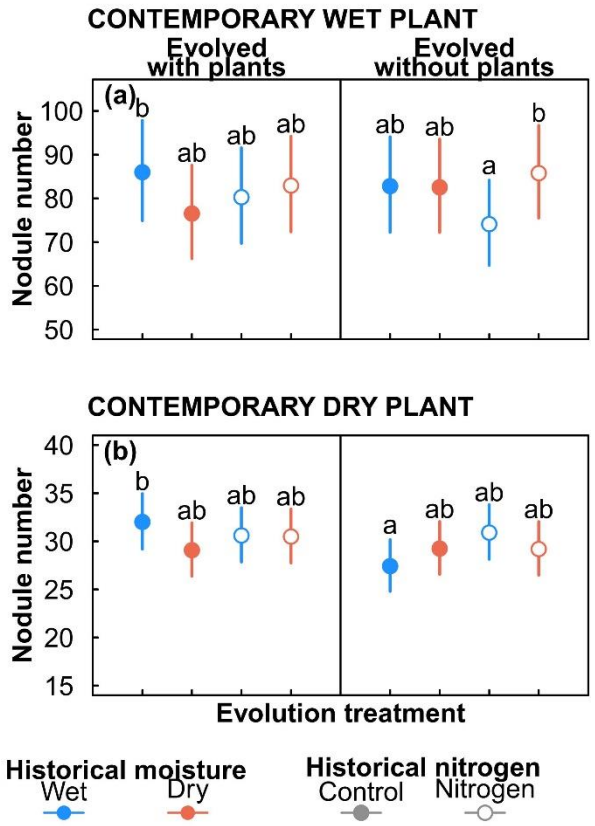

Table C.1: We generated principal components axes using the 3 aboveground traits to capture all our measures of plant health in a single measure. Separate measures were generated for the wet and dry treatments. Below we display the loadings of each of these traits into each of the first axes. We used PC1 to correlate with nodule number (see Figure 4).

Trait	**Wet treatment- PC1 loadings**	**Dry treatment- PC1 loadings**
Aboveground biomass	0.627	0.676
Plant height	0.540	0.507
Leaf number	0.562	0.534

Table C.2: Mixed model ANOVAs for the effects of the historic watering treatment (wet vs. dry), historic plant treatment (present vs. absent), historic nitrogen treatment (control vs. nitrogen), and their interactions on multiple traits associated with plant health, including above- and belowground biomass, leaf number, as well as height. We additionally show a MANOVA that combines all 4 plant traits together. These models are split by the contemporary watering treatment, and represent a splitting of the models reported in Table 1. We additionally display two auxiliary traits, displayed on the second half of this table, namely root-shoot ratio and nodule number. We display both the F value for each model term. Terms with an associated p value less than 0.05 are bolded. + $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Plant Traits											
Model Term	**df**	**Aboveground**	**Belowground**	**Height**	**Leaves**	**MANOVA**	**Aboveground**	**Belowground**	**Height**	**Leaves**	**MANOVA**
Historic Water	1	0.00	2.07	0.63	0.05	1.00	**7.11****	0.13	1.81	0.08	**5.11*****
Historic Plant	1	0.22	0.22	0.86	1.54	2.24^{+}	3.55^{+}	1.165	**10.56****	0.61	**5.32*****
Historic Nitrogen	1	0.05	0.58	1.22	1.74	1.55	0.02	0.90	1.21	0.24	0.44
HistWater x HistPlant	1	0.56	**3.93***	0.34	**4.27***	**4.54****	**4.08***	0.80	**7.64****	0.33	**3.68****
HistWater x HistNitro	1	3.80^{+}	1.21	3.54^{+}	**4.85***	1.97^{+}	0.39	0.04	0.37	0.83	0.83
HistPlant x HistNitro	1	**5.29***	0.78	**5.94***	2.69	**2.48***	0.96	3.04^{+}	1.17	0.21	1.37
HistPlant x HistWater x HistNitro	1	0.10	0.41	**8.96****	1.37	**3.60****	0.55	0.22	**5.87***	0.94	2.03^{+}
Auxiliary Traits											
Model Term	**df**	**Root-Shoot**	**Nodule Num.**				**Root-Shoot**	**Nodule Num.**			
Historic Water	1	1.04	0.06				**5.80***	0.20			
Historic Plant	1	0.68	0.03				**12.02*****	2.15			
Historic Nitrogen	1	0.72	0.14				1.14	0.76			
HistWater x HistPlant	1	2.18	3.00^{+}				**8.95****	0.42			
HistWater x HistNitro	1	1.75	**4.70***				0.41	0.10			
HistPlant x HistNitro	1	1.59	0.29				0.82	0.86			
HistPlant x HistWater x HistNitro	1	0.58	0.00				0.32	2.56			

Table C.3: Outcome of models, under both contemporary wet and dry conditions, correlating a composite measure of plant health (principal component axis 1, see Table C1) with nodule number. These models correspond to data displayed in Figure 4. As we were interested in variation in this correlation between the historical selective treatments, we included these treatments and their interactions with nodule number into the model. Variation between treatments in the correlation would be represented by a significant interaction term involving nodule number: we have colored such terms in the table for emphasis. We display the F value for each model term. Terms with an associated *p* value less than 0.05 are bolded. + $p \leq 0.1$; * $p \leq 0.05$; ** $p \leq 0.01$; *** $p \leq 0.001$.

Model Term	Contemporary Wet	Contemporary Dry
Nodule Number	**11.21*****	**50.86*****
Historical Nitrogen	**4.21***	1.60
Historical Water	0.45	3.81^{+}
Historic Plant	1.80	5.22*
NodNum x HistNitro	0.00	1.25
NodNum x HistWater	**7.05****	**17.14*****
NodNum x HistPlant	0.14	1.59
HistNitro x HistWater	3.36^{+}	0.01
HistNitro x HistPlant	**7.63****	2.52
HistWater x HistPlant	0.27	**4.73***
NodNum x HistNitro x HistWater	0.08	2.68
NodNum x HistNitro x HistPlant	0.20	0.28
NodNum x HistWater x HistPlant	0.32	**6.81****
HistNitro x HistWater x HistPlant	**6.21***	**4.97***
NodNum x HistNitrog x HistWater x HistPlant	0.94	0.09